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Most prostate cancer patients respond initially to androgen ablation and antiandrogen therapy. However, virtually all patients will relapse due to acquisition of the growth of the androgen-independent tumor cells. The molecular mechanism characterizing prostate cancer progression from androgen-dependence to androgen-independence is incompletely understood. We propose that Signal Transducers and Activators of Transcription 3 (Stat3) both regulates the expression of Stat3 target genes, and interacts with AR in prostate cancer cells. The experiments proposed in this application are based upon the hypothesis that Stat3 activation alters androgen receptor signaling pathways, that in turn results in the loss of growth control in prostate cancer cells. We propose to determine the consequence of Stat3 activation in prostate cancer cell growth and to determine the molecular basis of Stat3 interactions with androgen receptor signaling.

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Introduction

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Body

Since the approval of this application, we have made significant progress of task 1 (i.e., to examine the role of Stat3 activation in prostate cancer cells (months 1-18).

Task 1A. To establish a series of prostate cancer cell lines demonstrating constitutive Stat3 activation (months 1-6). We have established Stat3 constitutively activated cell sublines in LNCaP cells. These cells express activated Stat3 as examined by EMSA using Stat3 consensus binding sequences (reference 1).

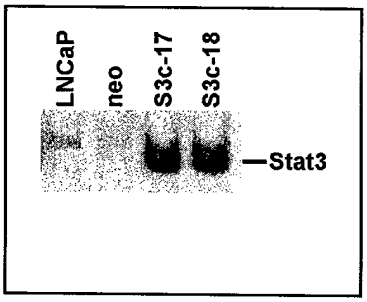
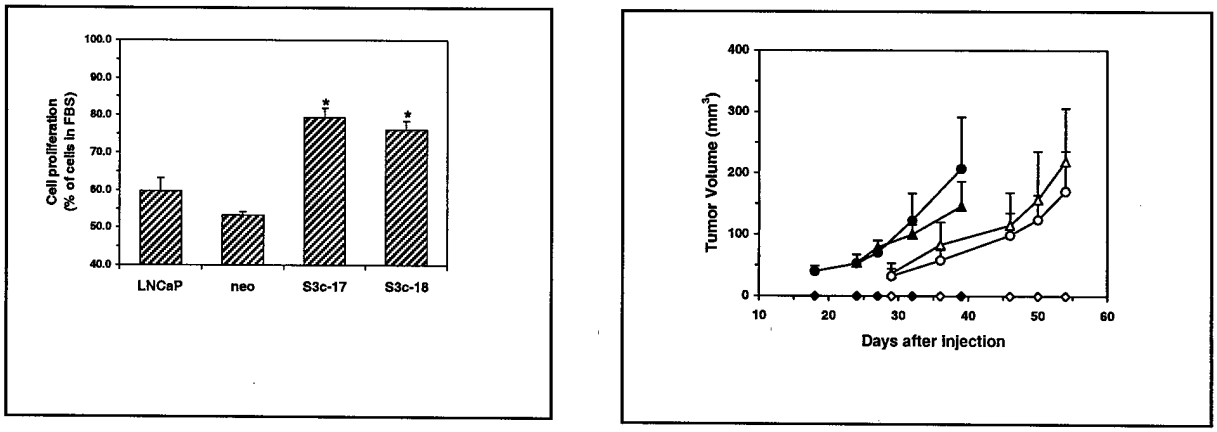


Fig 1. Stat3 DNA-binding activity in LNCaP and LN series cell lines. Whole cell extracts (20 μg) were subjected to EMSA using a ^{32}P-labeled oligonucleotide probe containing the consensus binding motif for Stat3.

Task 1B. To examine the effect of Stat3 activation on these cell growth in vitro and in vivo (months 7-12). We demonstrated that cells expressing constitutively activated Stat3 can enhance LNCaP androgen independent growth in vitro as analyzed by MTT assay (Fig 2A) and LNCaP growth in the castrated nude mice (Fig 2B), suggesting that Stat3 can enhance androgen independent growth of androgen-dependent LNCaP cells (reference 1).



A B

Figure 2. Stat3 enhances androgen-independent growth *in vitro*. **(A)** Effect of Stat3 on LNCaP cell growth in the presence and absence of androgen *in vitro*. Cells were cultured in RPMI-1640 supplemented with either 10% FBS or 10% charcoal-stripped FBS. Cell proliferation values in charcoal-stripped FBS were expressed as % relative to the complete FBS. *, $P < 0.05$. **(B)** Stat3 induces androgen-independent growth *in vivo*. Tumor growth curve in the intact and castrated male nude mice. Parental LNCaP cells and neo clone (◇) or clones that overexpress activated Stat3 (S3c-17, ∇; S3c-18, O) were injected into the intact (filled symbols) or castrated (open symbols) male nude mice (n = 10 for each condition).

Task 1C. To examine the effect of Stat3 activation on the expression of Stat3 target genes and AR-mediated genes (months 7-18). Prostate specific antigen (PSA) is a typical AR-mediated gene. We demonstrated that Stat3 can enhance PSA express both in mRNA levels as examined by Northern blot (Fig 3A) and in protein levels as examined by ELISA (Fig 3B). In addition, Stat3 can enhance PSA promoter activity and ARE-containing gene transactivation (Fig 4 and reference 1).

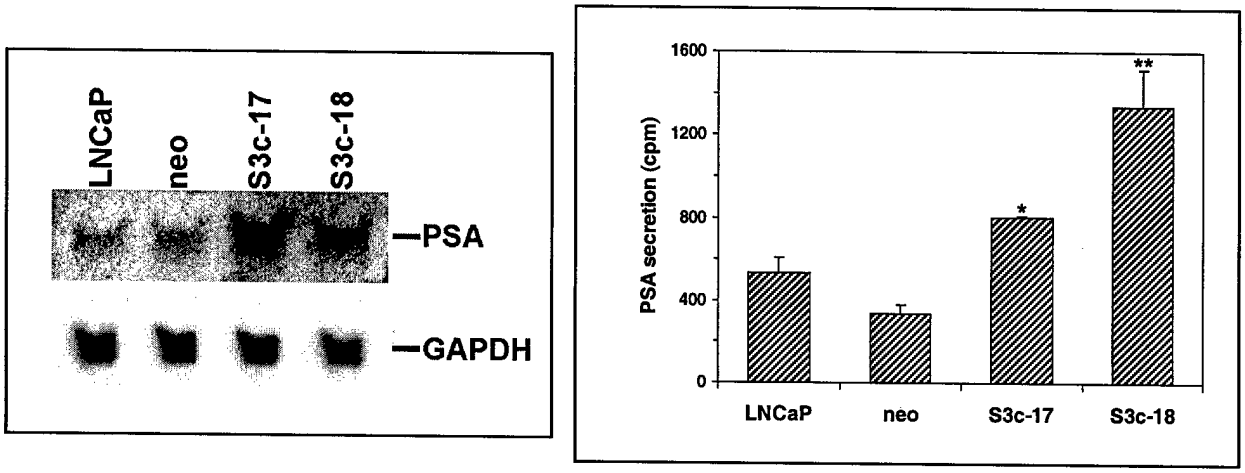


Figure 3. Stat3 enhances PSA expression. (A) PSA mRNA expression in Stat3 overexpressing clones (S3c-17, S3c-18), vector control (neo), and LNCaP cells examined by Northern blot analysis using 20 μg of total RNA. GAPDH is a control for equal loading. (B) PSA protein secretion in the absence of androgen. PSA secretion was quantitated by PSA immunoradiometric assay of 50 μl of supernatant of cell culture in phenol red-free RPMI containing 10% charcoal-stripped serum. *, $P < 0.05$; **, $P < 0.01$.

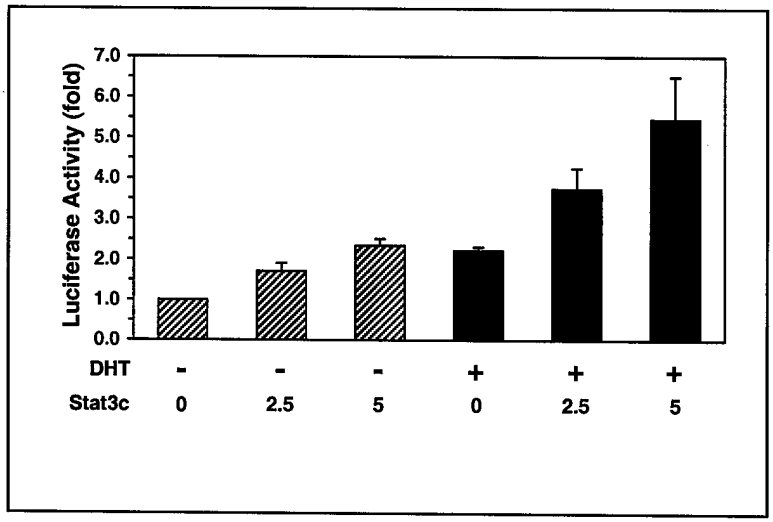


Figure 4. (A) Effect of Stat3 on PSA promoter activity in the absence of DHT and in the presence of 10 nM of DHT. LNCaP cells were transiently transfected with PSA-luc reporter, and increasing doses (0, 2.5, 5 μg) of Stat3 expression plasmid. Total DNA content was kept constant in all wells. HeLa cells transiently transfected with or without AR expression plasmid, PSA-luc reporter, and increasing doses (0, 2.5, 5 μg) of Stat3c expression plasmid in the presence of 10 nM of DHT. Total DNA content was kept constant in all wells. The luciferase activity was measured. Results are displayed as the average of four independent experiments. RLU: relative light units.

Task 2. To determine whether Stat3 activation induce androgen-independent growth by affecting AR signaling in prostate cancer cells (months 19-36).

We are initiating the experiments to study the interaction between Stat3 and AR and the consequences of this interaction in promoting androgen-independent prostate cancer. The experiments were designed to investigate the potential use of RNA interference to block Stat3 expression and activation and the effect on the growth of human prostate cancer cells. We identified a small interfering RNA (siRNA) specific for Stat3 and expressed in human prostate cancer cells from DNA expression vector which employ RNA polymerase III promoters from the U6 small nuclear RNA gene to transcribe siRNAs. We demonstrate that blockade of Stat3 activation by the Stat3 siRNA suppresses the growth of human prostate cancer cells and Stat3-mediated gene expression and induces apoptotic cell death. The Stat3 siRNA does not inhibit the proliferation nor induces apoptosis of Stat3-inactive human prostate cancer cells. In addition, the Stat3

siRNA inhibits the levels of AR-mediated gene- prostate specific antigen (PSA) expression in prostate cancer cells.

In addition, we have examined the potential of tyrosine kinase inhibitors to block Stat3 activation in prostate cancer cells. We evaluated two Stat3 signaling inhibitors: AG490 and JSI-124(Cucurbitacin-I). AG490 is a tyrosine kinase inhibitor selective for the JAK family kinases. We evaluated the effects of AG490 on the growth of prostate cancer cells that express activated Stat3 in vitro and in vivo. We chose to study the following human: LNCaP, DU145, PC3, CWR22Rv1 and Dunning rat: G, AT1, AT2, AT6.1, AT3.1, MatlLLu prostate cell lines. AG490 abrogated Stat3 activity and inhibited the growth of human and prostate cancer cell lines with an IC_{50} from 20-40 uM. To study the effects in vivo, we inoculated DU145 cells subcutaneously into the flanks of male nude mice. Once tumors reached a size of $0.5cm^3$ they either received AG490 (0.5mg/mice i.p daily for 14 days) or vehicle (DMSO/RPMI) only. AG490 suppressed tumor growth by 50% ($p<0.01$). AG490 administration did not cause any toxicity. JSI-124 is a plant natural product. We studied the effects of JSI-124 in human prostate cancer cell lines: LNCaP, DU145, PC3 and CWR22Rv1.We established the IC_{50} to range 0.5 to 1.0 uM. Our data suggest that targeting IL-6/Stat3 signaling may represent an opportunity in the development of new treatments for AIPC.

Androgen ablation induces apoptotic death of prostate epithelial cells and is a standard treatment for prostate cancer. However, androgen-independent prostate cancer cells become resistant to apoptosis, rendering androgen ablation therapy ineffective. To understand the role of Stat3 in androgen independent prostate cancer, we investigated the role of Stat3 activation in IL-6-mediated antiapoptotic activity in prostate cancer cells. We demonstrate that overexpression of IL-6 renders androgen sensitive LNCaP human prostate cancer cells more resistant to apoptosis induced by androgen deprivation. LNCaP cells undergo apoptosis after 72 h of androgen deprivation, an outcome is largely absent in clones overexpressing IL-6 as measured by cell death ELISA and chromatin degradation assays. IL-6 over-expressing cells resulted in a significant decrease in the expression of pro-apoptotic proteins such as cleaved PARP and cleaved caspase-9 as well as an increase in the expression of anti-apoptotic proteins Bcl-x_L and phosphorylated Bad. Addition of IL-6 antibody completely abolished the anti-apoptotic activity of IL-6. This protective effect of IL-6 was reversed by the expression of a dominant-negative Stat3 mutant, Stat3F. Furthermore, ectopic expression of a constitutively active Stat3 antagonized androgen deprivation-induced cell death of LNCaP cells. These results indicate that IL-6 protects androgen sensitive LNCaP cells from apoptosis induced by androgen deprivation, and Stat3 activation play an important role in IL-6-mediated anti-apoptosis in prostate cancer cells.

Key research accomplishments

- We demonstrated that Stat3 plays a critical role in prostate cancer growth.
- Stat3 enhances AR-mediated gene expression such as PSA.
- Stat3 enhances androgen independent growth of prostate cancer cells *in vitro* and *in vivo*.

- Stat3 activates androgen receptor (AR) in the presence and in the absence of androgen.
- Stat3 activation is required for IL-6 mediated antiapoptic activity in prostate cancer cells.
- Targeting Stat3 signaling by siRNA or tyrosine kinase inhibitors inhibits growth and induces apoptosis of prostate cancer cells.

Reportable outcome

Publications:

1. Lee SO, Lou W, Qureshi KM, Mehraein-Ghomi F, Trump DL, and **Gao AC**. RNA interference targeting Stat3 inhibits growth and induces apoptosis of human prostate cancer cells. Prostate, 60(4):303-9, 2004.
2. Lee SO, Lou W, Johnson CS, Trump DL, and **Gao AC**. Interleukin-6 protects LNCaP cells from apoptosis induced by androgen deprivation through the Stat3 pathway. Prostate 60: 178-186 , 2004.
3. Xie TX, Wei D, Liu M, **Gao AC**, Ali-Osman F, Sawaya R, Huang S. Stat3 activation regulates the expression of matrix metalloproteinase-2 and tumor invasion and metastasis. Oncogene 23(20):3550-60, 2004.
4. Dong Y, Lee SO, Zhang H, Marshall J, **Gao AC** and Ip C. Prostate specific antigen (PSA) expression is down-regulated by selenium through disruption of androgen receptor signaling. Cancer Res. 64 (1): 19-22, 2004, (Gao, AC, corresponding author).
5. Lee S.O., Lou W., Hou, M., Onate S.A., and **Gao A.C.** Interleukin-4 enhances prostate specific antigen expression by activation of the androgen receptor and Akt pathway. Oncogene 22 (39), 6037-6044, 2003.
6. Lee SO, Lou W, Demiguel F, Hou M, **Gao AC.** Interleukin-6 promotes androgen-independent growth in LNCaP human prostate cancer cells. Clin. Cancer Res., 9: 370-376, 2003.
7. Demiguel F, Lee S.O., Onate A. A., and **Gao AC.** Stat3 enhances transactivation of steroid hormone receptors. BioMed Central, Nuclear Receptor, 1:3, 2003.
8. Demiguel F, Lee S.O., Onate A. A., and **Gao AC.** Stat3 enhances transactivation of steroid hormone receptors. BioMed Central, Nuclear Receptor, 1:3, 2003.
9. Leman ES, DeMIGUEL F, **Gao AC**, Getzenberg RH. Regulation of Androgen and Vitamin D Receptors by 1,25-Dihydroxyvitamin D3 in Human Prostate Epithelial and Stromal Cells. J Urol. 170: 235-240, 2003.
10. Wei D, Le X, Zheng L, Wang L, Frey JA, **Gao AC**, Peng Z, Huang S, Xiong HQ, Abbruzzese JL, Xie K. Stat3 activation regulates the expression of vascular endothelial growth factor and human pancreatic cancer angiogenesis and metastasis. Oncogene 23;22(3):319-29, 2003.
11. DeMigeul F., Lou W., Lee S. O., Xiao X., Pflug B., Nelson J.B., and **Gao A.C.** Stat3 enhances the growth of LNCaP human prostate cancer cells in intact and castrated male nude mice. The Prostate, 52: 1-7, 2002.

Conclusions

We demonstrated that activation of Stat3 in androgen-sensitive LNCaP prostate cancer cells results in enhancement of tumor growth in both intact and castrated male nude mice, and enhances androgen receptor-mediated prostate specific antigen (PSA) expression. These findings demonstrate that intracellular signaling mediated by Stat3 can enhance the growth of androgen-sensitive human LNCaP prostate cancer cells in both intact and castrated male nude mice. Targeting Stat3 signaling by siRNA may serve as a novel therapeutic approach for prostate cancer.

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12. Lee SO, Lou W, Qureshi KM, Mehraein-Ghomi F, Trump DL, and **Gao AC**. RNA interference targeting Stat3 inhibits growth and induces apoptosis of human prostate cancer cells. Prostate, 60(4):303-9, 2004.
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Appendices

- Lee SO, Lou W, Qureshi KM, Mehraein-Ghomi F, Trump DL, and **Gao AC**. RNA interference targeting Stat3 inhibits growth and induces apoptosis of human prostate cancer cells. Prostate, 60(4):303-9, 2004.

- Lee SO, Lou W, Johnson CS, Trump DL, and **Gao AC**. Interleukin-6 protects LNCaP cells from apoptosis induced by androgen deprivation through the Stat3 pathway. Prostate 60: 178-186 , 2004.

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The Prostate 60:303–309 (2004)

Appendix 1

RNA Interference Targeting Stat3 Inhibits Growth and Induces Apoptosis of Human Prostate Cancer Cells

Soo Ok Lee, Wei Lou, Khusroo M. Qureshi, Farideh Mehraein-Ghomi, Donald L. Trump, and Allen C. Gao*

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Stat3, a member of the signal transduction and activation of transcription (STAT) family, is a key signal transduction protein that mediates signaling by cytokines, peptide growth factors, and oncoproteins and is constitutively activated in numerous cancers including prostate. Previous studies demonstrated that constitutively activated Stat3 plays an important role in the development and progression of prostate cancer by promoting cell proliferation and protecting against apoptosis. The present study was designed to investigate the potential use of RNA interference to block Stat3 expression and activation and the effect on the growth of human prostate cancer cells. We identified a small interfering RNA (siRNA) specific for Stat3 and demonstrate that blockade of Stat3 activation by the Stat3 siRNA suppresses the growth of human prostate cancer cells and Stat3-mediated gene expression and induces apoptotic cell death. The Stat3 siRNA does not inhibit the proliferation nor induces apoptosis of Stat3-inactive human prostate cancer cells. In addition, the Stat3 siRNA inhibits the levels of androgen-regulated prostate specific antigen (PSA) expression in prostate cancer cells. These results demonstrate that targeting Stat3 signaling using siRNA technique may serve as a novel therapeutic strategy for treatment of prostate cancer expressing constitutively activated Stat3. *Prostate 60: 303–309, 2004.* © 2004 Wiley-Liss, Inc.

KEY WORDS: **Stat3; RNA interference; prostate cancer; apoptosis**

INTRODUCTION

Stat3, a member of the signal transduction and activation of transcription (STAT) family, is a key signal transduction protein that mediates signaling by numerous cytokines, peptide growth factors, and oncoproteins [1]. Accumulating evidence demonstrates that Stat3 activation plays important roles in cell differentiation, proliferation, development, apoptosis, and inflammation [2]. Elevated activity of Stat3 has been found frequently in a wide variety of human tumors, including hematologic malignancies, head and neck, breast and prostate cancer [2]. Cell lines from multiple myelomas that have become growth factor independent require constitutively active Stat3 to protect against apoptosis [3]. In addition, constitutively activated Stat3 induces cellular transformation in vitro and tumor formation in nude mice [4].

Studies to date provide strong evidence that aberrant Stat3 signaling plays an important role in the development and progression of prostate cancer by promoting cell proliferation and protecting against apoptosis. Stat3 activity is not only significantly increased in human primary prostate cancer tissues as compared with normal, but it is also increased in androgen independent prostate cancer cells as compared to androgen sensitive cells [5,6]. Stat3 is a major mediator of interleukin-6 (IL-6) induced signaling in prostate cancer cells and that IL-6 induced androgen

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receptor (AR)-mediated gene activation requires the activation of Stat3 in LNCaP prostate cancer cells [7,8]. Constitutive activation of Stat3 promotes androgen independent growth of androgen dependent LNCaP cells in vitro and in vivo [9]. Blockade of Stat3 expression in human prostate cancer cells suppress proliferation in vitro and tumorigenicity in vivo [10]. These studies demonstrate that constitutively activated Stat3 is not only associated with prostate cancer, but also induces prostate cancer cell proliferation. Thus, the Stat3 signaling pathway may represent a new molecular target for novel therapeutic approaches for prostate cancer. Strategies that target Stat3 signaling have been proposed including tyrosine kinase inhibitors such as tyrphostin AG490 and cucurbitacin I [11,12], antisense oligonucleotides and decoy oligonucleotides [6,13], and dominant-negative Stat3 protein [10,14].

Small interfering RNA (siRNAs) are short double-stranded RNA molecules that can target complementary mRNAs for degradation via a cellular process termed RNA interference (RNAi) [15]. RNAi is usually activated by introducing long double-stranded RNA molecules into cells which are cleaved into 21- to 23-nt RNAs referred to as siRNAs by an endonuclease named Dicer in animal cells [15]. The siRNA molecules then serve as a guide for sequence-specific degradation of homologous mRNAs. SiRNA has been used for functional analysis of genes in many species including invertebrates, plants, and mammalian cells [16]. Recently, siRNA has emerged as powerful RNAi reagents for directed posttranscriptional gene silencing and inhibition of viral propagation [17]. The potential of using siRNA for silencing specific genes has been demonstrated in treatment of viral diseases and cancer including HIV, human papillomavirus, and hepatitis C virus [18].

The present study was designed to investigate the potential use of siRNA to block Stat3 expression and the effect on growth of human prostate cancer cells. We identified a siRNA specific for Stat3 and expressed in prostate cancer cells. We demonstrate that blockade of Stat3 expression by this siRNA inhibits the growth of human prostate cancer cells and induces apoptotic cell death. These results demonstrate that targeting Stat3 signaling using siRNA technique may serve as a novel therapeutic strategy for treatment of prostate cancer expressing constitutively activated Stat3.

MATERIALS AND METHODS

Tissue Culture

Human DU145 prostate cancer cells were maintained in RPMI1640 supplemented with 10% of FBS. LN-17 cells were generated from human LNCaP prostate cancer cells stably expressing IL-6 as described previously [8,19], and cultured in RPMI 1640 supplemented with 10% FBS plus 0.3 mg/ml of G418. Human PC3 prostate cancer cells were maintained in DMEM supplemented with 10% FBS. The cells were cultured in 100 U/ml of penicillin and 100 μg/ml of streptomycin at 37°C in 5% CO_2 incubator.

Plasmids and siRNA Expression

The oligonucleotides containing sequences specific for Stat3 (5′-GATCCCGTGTTCTCTATCAGCACAA-TTTCAAGAGAATTGTGCTGATAGAGAACATTTT-TTGGAAA-3′ and 5′-AGCTTTTCCAAAAAATGTTC-TCTATCAGCACAATTCTCTTGAAATTGTGCTGA-TAGAGAACACGG-3′) were synthesized and annealed. Stat3 siRNA expression vector that express hairpin siRNAs under the control of the mouse U6 promoter was constructed by inserting pairs of the annealed DNA oligonucleotides into the pSilencer-neo siRNA expression vector that was digested with BamHI and HindIII (Ambion, Austin, TX). A pSilencer neo vector that expresses a hairpin siRNA with limited homology to any known sequences in the human, mouse, and rat genomes was used as a negative control (Ambion). The negative control siRNAs have been tested in multiple cell lines and they show no toxicity to cells when analyzed by Trypan Blue staining and cell counting 48 hr after transfection. The negative control siRNAs have no effect on the expression of the mRNA levels of "housekeeping" genes, including 28S rRNA, GAPDH, and Cyclophilin (Ambion).

Stat3 siRNA Transfection

DU145, PC3, and LN-17 cells were transiently transfected either with Stat3 siRNA or the negative control plasmid using SuperFect Transfection Reagent (QIAGEN, Inc., Valencia, CA). Briefly, cells were plated into either 12-well plates (10^5 cells per well) or 100 mm dish (10^6 cells) and allowed to adhere for 24 hr. Cells were transfected with either Stat3 siRNA expression vector or negative control siRNA plasmids in serum-free medium for 4 hr, incubated with complete medium for 24 hr, and then cells were switched into medium containing G418 (0.3 mg/ml). Two days later, protein extracts and total RNA were prepared for subsequent analysis.

In Vitro Growth Assay

DU145, PC3, and LN-17 cells (1×10^5 per well) were plated in 12-well plates in RPMI containing 10% FBS. Cells were transfected with either Stat3 siRNA expression vector or negative control plasmid as described above. Four days later, cells were counted with a Coulter counter.

Apoptosis ELISA Test

Cell death detection ELISA kit (catalog # 1544675) was obtained from Roche Molecular Biochemicals (Indianapolis, IN) and the protocol was followed according to the manufacturer's instructions. For the preparation of samples, cells were plated and transfected with either 1 μg of Stat3 siRNA expression vector or 1 μg of control plasmid as described previously. Three days later, cell lysates were obtained and cell death was analyzed and the absorbance values were normalized by the amount of protein. The protein concentration was determined by Bradford assay (Coomassie Plus, Pierce, Rockford, IL).

Luciferase Assay

DU145 cells were plated (1×10^5 cells per well of 12-well plate) and transfected with 1 μg of pLucTKS3 reporter plasmid containing specific responsive elements for Stat3 [20], varied amount of Stat3 siRNA expression vector or negative control vector. For control, DU145 cells were cotransfected with 1 μg of Stat3 unresponsive pLucTK reporter plasmid [20] and Stat3 siRNA expression vector or negative control vector. Total amount of DNA transfected was adjusted using empty vector DNA. After 40 hr of incubation, cell extracts were prepared and luciferase activity was determined according to manufacturer's protocols (Promega, Madison, WI). Luciferase activity was normalized per microgram of protein as determined by Bradford assay (Coomassie Plus, Pierce).

Electromobility Shift Assay

After transfection with either Stat3 siRNA expression vector or negative control plasmid, nuclear extracts were prepared and electromobility shift assay (EMSA) was performed as described previously [10]. For determination of the Stat3 DNA binding activity, nuclear extracts (10 μg) were incubated in a final volume of 20 μl (10 mM HEPES, pH 7.9, 80 mM NaCl, 10% glycerol, 1 mM DTT, 1 mM EDTA, 100 μg/ml poly(dI-dC) with radio labeled double stranded Stat3 consensus binding motif 5′-GATCCTTCTGGGAA-TTCCTAGATC (Santa Cruz Biotechnologies, CA). The protein–DNA complexes were resolved on a 4.5% non-denaturing polyacrylamide gel at room temperature, and the results were autoradiographed using Molecular Imager FX System (Bio Rad, Hercules, CA).

Western Blot Analysis

Cell extracts were prepared and resolved on a 10% SDS–PAGE and blotted onto a membrane. After blocking overnight at 4°C in 5% milk in PBS containing 0.1% Tween 20, membranes were incubated overnight with either antibodies of cleaved poly (ADP-ribose) polymerase (PARP) (Cell Signaling Technology, Beverly, MA), Bcl-x_L (Santa Cruz, CA), or antibodies against Stat3 or phospho-Stat3 proteins (Cell Signaling Technology). Following secondary antibody incubation, immunoreactive proteins were visualized with an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Buckinghamshire, England).

Northern Blot Analysis

Twenty micrograms of total RNAs were electrophoresed in 1.2% denaturing agarose gels and transferred to a nylon membrane (MSI, Westborough, MA). PSA cDNA and Stat3 cDNA were labeled with [α-^{32}P] dCTP (3,000 Ci/mmol, ICN, Costa Mesa, CA) using the Ready-To-Go DNA Labeling Beads (Amersham Pharmacia Biotech). Hybridization was carried out during 3 hr at 65°C in Rapid-hyb buffer (Amersham Pharmacia Biotech). Membranes were washed for 15 min at 65°C in 2× SSC, 0.1% SDS (twice), 0.5× SSC, 0.1% SDS, and 0.1× SSC, 0.1% SDS. Radioactivity in the membranes was analyzed with a Molecular Imager FX System (Bio Rad).

PSA Protein Analysis

PSA secretion was quantitated by ELISA with the use of anti-PSA as primary antibody as described by the manufacturer's protocol (Beckman Coulter, Fullerton, CA). Equal numbers of cells were plated in phenol red-free RPMI containing 10% FBS. Cells were allowed to attach for 24 hr, then the cells were transfected with either 1 μg of Stat3 siRNA or 1 μg of control siRNA. After 3 days, 50 μl of supernatant was assayed for PSA.

Statistical Analysis

Values were expressed as the mean ± SE. Statistical analyses were performed by one-way ANOVA, followed by the Student–Newman–Keuls test for multiple comparisons, with a $P < 0.05$ significant.

RESULTS

Stat3 siRNA Inhibits Stat3 DNA-Binding Activity

To determine whether transfection of Stat3 hairpin siRNA vector inhibits Stat3 activity, we used several human prostate cancer cell lines expressing high levels of constitutively activated Stat3 including DU145 and LN-17 cells [8,10]. The LN-17 cells were derived from LNCaP cells that were ectopically transfected with IL-6 cDNA and expressed constitutively activated Stat3 [8,19]. Both cell lines were transfected with either the Stat3 hairpin siRNA vector or the negative control siRNA vector, respectively. Antibiotic G418 (300 μg/ml) was added following 24 hr transfection and the cells

were allowed to grow for another 48 hr. The cells were harvested and the Stat3 activity was evaluated by EMSA. The Stat3 hairpin siRNA vector transfected cells demonstrated a marked decrease in formation of Stat3 DNA–protein complex in the gel shift assay compared to the negative control siRNA vector transfected cells (Fig. 1A).

We also examined the effect of Stat3 siRNA on the levels of endogenous Stat3 mRNA. Figure 1B shows that Stat3 siRNA significantly inhibits the steady-state expression levels of Stat3 mRNA compared to that of negative control siRNA vector.

To determine whether the Stat3 siRNA affect the levels of steady-state Stat3 or phosphorylated Stat3 protein, we performed Western blot analysis using antibodies against either Stat3 or phospho-specific Stat3 protein on the cells transfected with either Stat3 siRNA expression vector or negative control siRNA vector. Figure 1C shows that both the levels of steady-state Stat3 and tyrosine-phosphorylated Stat3 protein were decreased by Stat3 siRNA compared to the negative control siRNA vector. It appears that the reduction in steady state levels of total and phosphorylated Stat3 is less than the Stat3-DNA binding activity and Stat3 mRNA levels by siRNA Stat3.

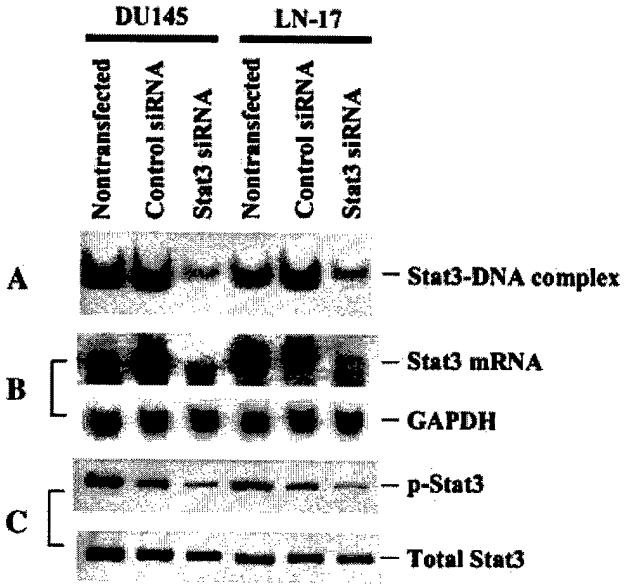

Fig. 1. Effect of Stat3 siRNA on Stat3 expression and activity of human prostate cancer cells. **A**: Stat3–DNA binding activity. The Stat3 activity was analyzed using EMSA as described in Materials and Methods. **B**: Stat3 mRNA levels of human prostate cancer cells transfected with Stat3 siRNA or control siRNA were determined by Northern blot analysis. GAPDH expression was used as an internal control. **C**: Effect of Stat3 siRNA on Stat3 protein expression. Western blots were performed using either antibodies against phospho-specific Stat3 (Tyr-705) or total Stat3 with whole cell extracts isolated from prostate cancer cells transfected with Stat3 siRNA expression vector or control siRNA vector.

Stat3 siRNA Inhibits Cell Growth and Induces Apoptosis

We have previously demonstrated that constitutively activation of Stat3 promotes human prostate cancer cell growth in vitro and tumor growth in vivo [9]. To determine the effect of the Stat3 siRNA on human prostate cancer cell growth, DU145 and LN-17 cells were transiently transfected with either Stat3 siRNA expression vector or the negative control vector. The cells were counted after 4 days of transfection. Figure 2A shows that Stat3 siRNA expression vector inhibited cellular proliferation by 60% in DU145 cells and 50% in LN-17 cells compared to that of the negative

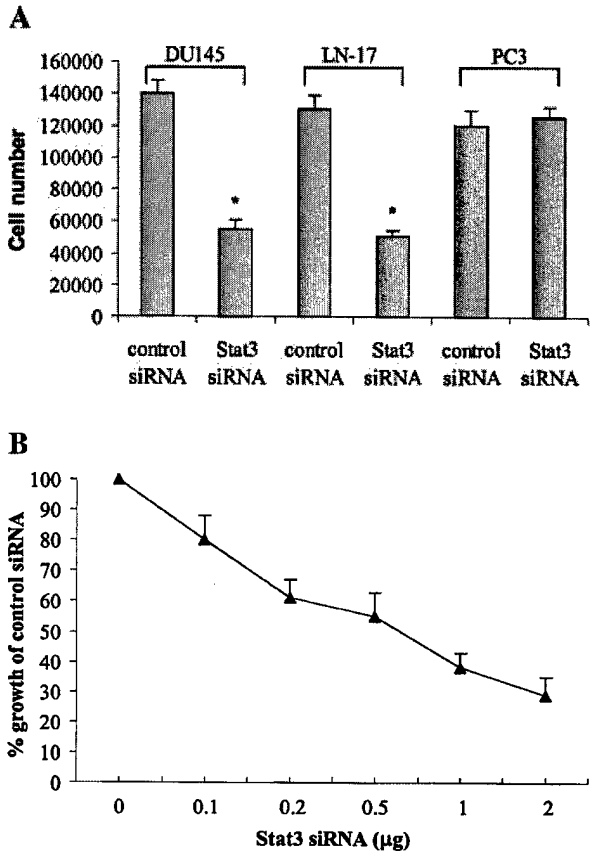

Fig. 2. Effect of Stat3 siRNA on human prostate cancer cell growth. **A**: Human prostate cancer DU145, PC3, and LN-17 cells (1×10^5 per well) were plated in 12-well plates in RPMI 1640 containing 10% FBS. After 24 hr, the cells were transfected with either Stat3 siRNA expression vector or negative control plasmid. Four days later, the cells were counted with a Coulter counter. Columns represent means of data ($n = 4$); bars, ±SE. *, Significantly different from control siRNA. **B**: Transfected with 0.1–2 μg Stat3 siRNA expression vector demonstrated a dose-dependent inhibition of proliferation of DU145 cells. The data are expressed as % of the control siRNA.

control vector. To determine whether the growth inhibition by Stat3 siRNA was dose-dependent, prostate cancer cells were transfected with a range of Stat3 siRNA expression vector (0.1–2 μg/well) and the cell number was determined afterwards. As shown in Figure 2B, the growth inhibition by the Stat3 siRNA was dose-dependent.

Activation of Stat3 protects cells from apoptotic cell death, and blockade of Stat3 activation induces apoptosis [3,6]. Therefore, we evaluated the effect of the Stat3 siRNA on apoptosis in human prostate cancer cells. The Stat3 siRNA induced apoptosis was examined using an apoptosis specific ELISA kit. Figure 3A shows that the Stat3 siRNA induced apoptosis in DU145 by threefold and in LN-17 by fourfold compared to the negative control vector. Immunoblots using antibodies against Bcl-x_L and cleaved PARP proteins were also performed to determine the effect of the Stat3 siRNA on apoptosis. As shown in Figure 3B, the Stat3 siRNA significantly reduced the expression of Bcl-x_L and enhanced the expression of cleaved PARP compared to the negative control vector, further demonstrating that the Stat3 siRNA induces human prostate cancer cells to undergo apoptotic cell death.

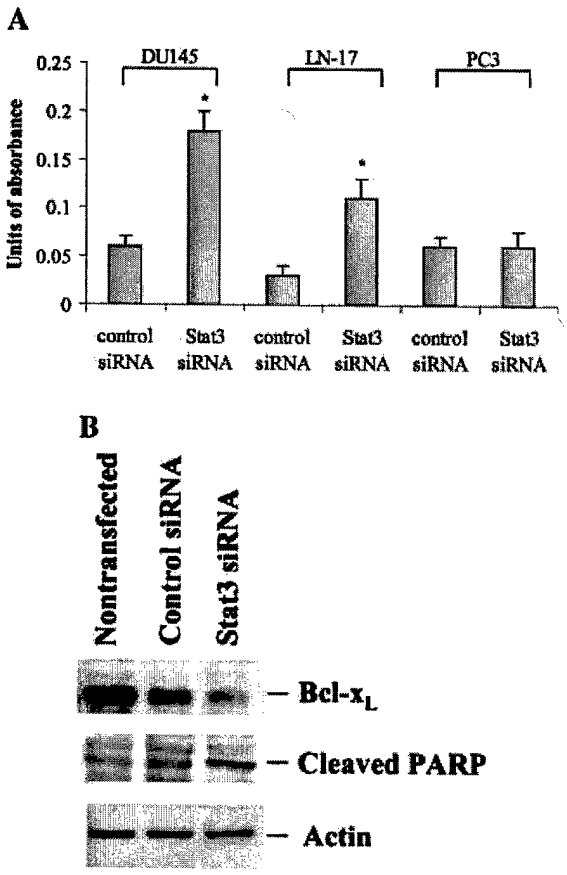

Fig. 3. Effect of Stat3 siRNA on prostate cancer cell apoptosis. **A**: Cell death analysis by a specific ELISA kit. Human prostate cancer DU145, LN-17, and PC3 cells were transfected with Stat3 siRNA expression vector or control siRNA vector. Three days later, cell lysates were obtained and cell death was analyzed and the absorbance values were normalized by the amount of protein. Data are expressed as mean ± SD of four independent experiments. *, Significantly different from control siRNA. **B**: Western blot analysis of the expression of Bcl-x_L and cleaved PARP proteins in LN-17 prostate cancer cells transfected with Stat3 siRNA expression vector or control siRNA vector. Immunoblots were prepared from 40 μg of whole cell lysate from LN-17 cells transfected with Stat3 siRNA expression vector or control siRNA vector.

We also evaluated the selectivity of the Stat3 siRNA action. We transfected Stat3 siRNA expression vector into Stat3-negative PC3 human prostate cancer cells. The Stat3 siRNA shows no effect on the proliferation (Fig. 2A) nor apoptosis (Fig. 3A) in PC3 cells compared to the negative control vector, suggesting that the Stat3 siRNA inhibits cell proliferation only in cells that express constitutively activated Stat3.

Stat3 siRNA Inhibits Stat3-Mediated Gene Expression

Stat3 activation contributes to oncogenesis through regulation of its target genes. To examine the effect of the Stat3 siRNA on Stat3-mediated gene expression, DU145 cells were cotransfected with the pLucTKS3 reporter containing the Stat3 responsive elements [20] and with either Stat3 siRNA expression vector or negative control vector, and cytosolic extracts were prepared for luciferase assays. The pLucTK reporter that contains no Stat3 responsive element was used as a control [20]. As shown in Figure 4A, the Stat3 siRNA significantly inhibited the induction of the Stat3-dependent pLucTKS3 luciferase reporter activity without affecting the Stat3-independent pLucTK activity compared to the negative control vector.

We previously demonstrated that activated Stat3 enhances the expression of AR-mediated genes including PSA [9]. To test the effect of the Stat3 siRNA on PSA expression, Northern blot analyses were performed to compare the levels of the expression of PSA mRNA in LN-17 cells that have been transfected with either Stat3 siRNA expression vector or negative control vector. As shown in Figure 4B,C, the Stat3 siRNA significantly reduced the levels of PSA expression compared to the negative vector control.

DISCUSSION

Studies to date provide strong evidence that aberrant Stat3 signaling may play an important role in the development and progression of prostate cancer. We previously demonstrated increase Stat3 activation in prostate cancer and that constitutively activated Stat3 promotes prostate cancer cell tumor growth [5,9].

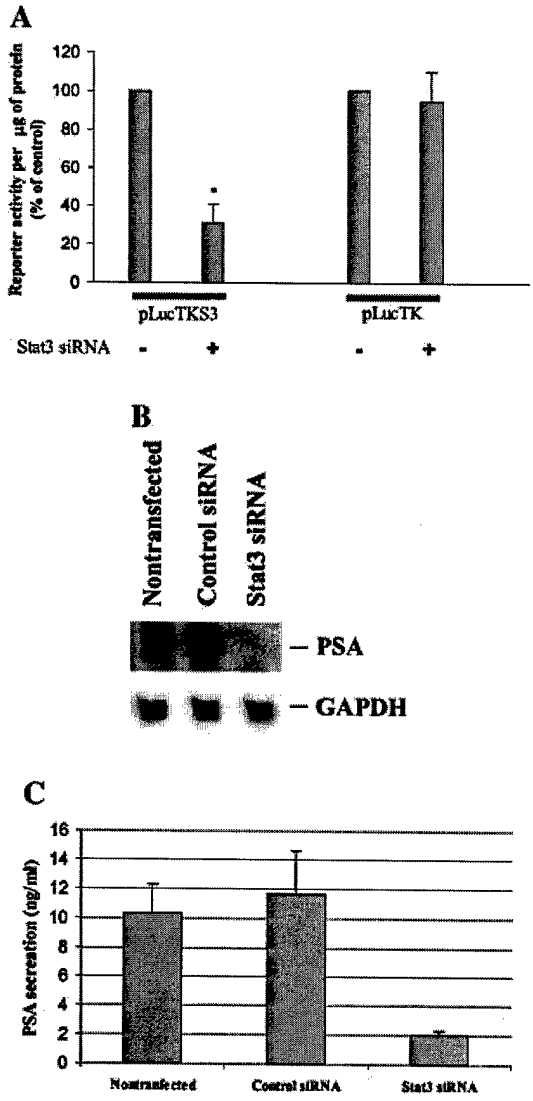

Fig. 4. Effect of Stat3 siRNA on Stat3-mediated gene expression. **A**: DU145 cells expressing constitutively activated Stat3 were cotransfected with the pLucTKS3 reporter (1 μg) containing the Stat3 responsive elements and with either 1 μg of Stat3 siRNA expression vector (+) or 1 μg of negative control vector (−). Cell extracts were prepared for luciferase assays. The pLucTK reporter that contains no Stat3 responsive element was used as a control. Luciferase activity was determined according to manufacturer's protocols and normalized per μg of protein. Data are representative of three independent experiments. *, Significantly different compared to control siRNA. **B**: Stat3 siRNA inhibits PSA mRNA expression in LN-17 cells. Total RNA was isolated from LN-17 cells transfected with either Stat3 siRNA expression vector or control siRNA vector and used for Northern blot analysis as described in Materials and Methods. GAPDH was used as a loading control. **C**: Stat3 siRNA inhibits PSA protein expression in LN-17 cells. The levels of PSA protein expression in the medium was analyzed by PSA ELISA from LN-17 cells transfected with either Stat3 siRNA expression vector or control siRNA vector.

Blockade Stat3 activation by a dominant negative Stat3 mutant resulted in suppression of prostate cancer growth both in vitro and in vivo [10]. Numerous studies also demonstrate that Stat3 activates AR-mediated gene expression and prevents cell from apoptosis [6,7,9]. Collectively, these findings indicate that targeting Stat3 signaling may represent a novel approach to treat prostate cancer.

RNAi represents a promising new technology that could have therapeutic applications for the treatment of diseases including cancer by blocking the action of transcription factors and oncogenes with selective silencing of gene expression with exquisite precision and high efficacy [21]. In this study, we have identified the Stat3 siRNA that specifically inhibits constitutively activated Stat3 and suppresses cell proliferation and induces apoptosis. To compare the relative potency of this siRNA Stat3 with Jak/Stat3 inhibitor AG490, LN-17 cells were transfected with 1 μg of Stat3 siRNA expression plasmid and the cell number was determined, while the same cells were treated with different doses of AG490 (range from 0 to 50 μM). The effect of inhibition of cell growth by expression of 1 μg of Stat3 siRNA (about 60% inhibition) is similar to the effect of 30 μM of AG490.

Previous studies demonstrated that Stat3 is constitutively activated in human prostate cancer compared to normal prostate [5,6], and that constitutively activated Stat3 promotes prostate cancer cell growth both in vitro and in vivo [9]. This study showed that the Stat3 siRNA only inhibits the proliferation and induces apoptosis in cells expressing constitutively activated Stat3, but not in Stat3-inactive PC3 cells, further demonstrating the selectivity of the Stat3 siRNA and potential therapeutic utility of the Stat3 siRNA for prostate cancer expressing active Stat3.

The PSA is synthesized primarily by normal and malignant prostate and the levels of PSA in serum correlate with the clinical stage of the disease. We have previously demonstrated that constitutively activated Stat3 enhances PSA expression in vitro and in vivo and enhances PSA transcription [9]. We showed here that blockade of Stat3 activation by the Stat3 siRNA significantly inhibited PSA mRNA expression in LN-17 cells (Fig. 4B), indicating that targeting Stat3 activation by the Stat3 siRNA could inhibit the AR-mediated gene expression in prostate cancer cells.

In conclusion, we have identified the Stat3 siRNA that specifically inhibits constitutively activated Stat3 and suppresses cell proliferation and induces apoptosis. The Stat3 siRNA resulted in blockade of Stat3 DNA-binding activity and inhibited the levels of Stat3 mRNA. Targeting Stat3 activation with RNAi holds therapeutic promise for prostate cancer with constitutive Stat3 activation. Several strategies have

been reported to block Stat3 activation including using Stat3 decoy oligonucleotides in head and neck cancer cells [13]. Although siRNA method has emerged as powerful RNAi reagents for directed posttranscriptional gene silencing and treatment of viral diseases and cancer, it would be of interest to compare the relative therapeutic potency of using Stat3 decoy oligonucleotides with the Stat3 siRNA identified in this study in prostate cancer cells.

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The Prostate 60:178 – 186 (2004)

Appendix 2

Interleukin-6 Protects LNCaP Cells From Apoptosis Induced by Androgen DeprivationThrough the Stat3 Pathway

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BACKGROUND. Elevated expression of interleukin-6 (IL-6) is implicated in the progression of hormone refractory prostate cancer. Previous studies demonstrated that IL-6 promotes androgen-independent growth of prostate cancer cells. In this study, the effect of IL-6 on apoptosis induced by androgen deprivation was investigated.

METHODS. The effect of IL-6 on apoptosis induced by androgen deprivation in LNCaP cells was examined by cell death ELISA and Western blot using cleaved poly (ADP-ribose) polymerase (PARP) and caspase-9, as well as Bcl-x_L and phosphorylated Bad. The Stat3 in IL-6-mediated anti-apoptosis in prostate cancer cells was examined using either dominant-negative or constitutively activated Stat3 mutants.

RESULTS. Overexpression of IL-6 renders androgen sensitive LNCaP human prostate cancer cells more resistant to apoptosis induced by androgen deprivation. LNCaP cells undergo apoptosis after 72 hr of androgen deprivation, an outcome is largely absent in clones overexpressing IL-6 as measured by cell death ELISA and chromatin degradation assays. IL-6 over-expressing cells resulted in a significant decrease in the expression of cleaved PARP and cleaved caspase-9 as well as an increase in the expression of Bcl-x_L and phosphorylated Bad. Addition of IL-6 antibody completely abolished the anti-apoptotic activity of IL-6. This protective effect of IL-6 was reversed by the expression of a dominant-negative Stat3 mutant, Stat3F. Furthermore, ectopic expression of a constitutively active Stat3 antagonized androgen deprivation-induced cell death of LNCaP cells.

CONCLUSION. These results indicate that IL-6 protects androgen sensitive LNCaP cells from apoptosis induced by androgen deprivation, and Stat3 activation play an important role in IL-6-mediated anti-apoptosis in prostate cancer cells. *Prostate 60: 178–186, 2004.*

KEY WORDS: **IL-6; prostate cancer; apoptosis; Stat3; androgen-independence**

INTRODUCTION

Prostate cancer is the most commonly diagnosed cancer and second leading cause of cancer death in American men. Initially, the growth of prostate epithelial cells is dependent on androgen. When androgen is depleted, these cells undergo apoptosis and die, the basis for androgen ablation therapy, a common treatment for prostate cancer [1,2]. However, most patients will relapse to hormone refractory disease due to the growth of androgen-independent cancer cells. In this stage, cells are more resistant to apoptotic cell death and thus androgen ablation is ineffective [2].

Interleukin-6 (IL-6) is a 21–28 Kd multifunctional cytokine involved in many cellular processes such as

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inflammation, cell differentiation, and proliferation [3]. IL-6 production correlates with tumor progression in human cancer such as pleural mesothelioma, glioblastoma, and ovarian and prostate cancer [4–7]. The expression of IL-6 and its receptor is consistently demonstrated in human prostate cancer cell lines and in freshly isolated human prostate carcinoma and benign prostate hyperplasia [8–10]. Clinically, the levels of IL-6 in serum are significantly elevated in many men with advanced, hormone-refractory prostate cancer [4,11]. IL-6 has been suggested to have both growth-promoting and inhibiting activities in androgen-sensitive LNCaP human prostate cancer cells in vitro [12–15]. IL-6 activates AR-mediated gene expression in LNCaP cells in vitro [14,16–18], suggesting that IL-6 may play a critical role during the progression of prostate cancer. In addition, overexpression of IL-6 in androgen sensitive LNCaP human prostate cancer cells promotes LNCaP cell androgen-independent growth in vitro and in vivo [19].

The biological activities of IL-6 are mediated by the IL-6 receptor. IL-6 receptor is composed of two components, a 80 Kd transmembrane protein that has ligand-binding capacity and a 130 Kd membrane glycoprotein (gp130) that mediates signal transduction following IL-6 binding [20]. The IL-6 receptor is abundant in many types of prostate cells including both androgen-dependent and androgen-independent cells [10]. In addition to the transmembrane IL-6R, a soluble IL-6 form of IL-6R (sIL-6R) can be generated either from truncated membrane protein or translation from an alternatively spliced mRNA [21]. IL-6 can bind to this soluble form of the receptor, an alternate target for the biologic activity [21]. IL-6 signaling through gp130 transduces signals into the interior of the cell through several major signaling pathways including the Janus kinase (JAK)-Signal Transducers and Activators of Transcription (STAT) pathway [22], mitogen-activated protein (MAP) kinase pathways [23], and the phosphatidylinositol (PI) 3-kinase-AKT pathways [24].

Apoptosis is a physiological cellular suicide program that maintains tissue homeostasis with pro-apoptotic and anti-apoptotic protein family members implicated in cell survival/death decisions. The role of IL-6 in the regulation of apoptosis is demonstrated in many cancer cells. Overexpression of IL-6 increases anti-apoptotic activity and thereby tumorigenic potency in basal cell carcinoma [25]. IL-6 can regulate the anti-apoptotic Bcl-2 family proteins, and the expression of Mcl-1, a Bcl-2 family member, was significantly induced by IL-6 [26].

While IL-6 plays a critical role in the development of androgen independent prostate cancer, the molecular mechanisms of IL-6 mediated androgen independence are largely unknown. In this study, we demonstrated that IL-6 can protect androgen-sensitive LNCaP human prostate cancer cells from apoptotic death induced by androgen depletion. Furthermore, the anti-apoptotic activity of IL-6 is mediated by Stat3 signaling pathway.

MATERIALS AND METHODS

Tissue Culture

The LNCaP cells were maintained in RPMI 1640 supplemented with 10% of FBS, 100 U/ml of penicillin, and 100 μg/ml of streptomycin at 37°C in 5% CO_2 incubator. Transfection and selection of LNCaP cells stably expressing IL-6 were described previously [13]. The clones overexpressing IL-6 (LN-S15, LN-S17) and its neo control (transfected with vector alone) cells were cultured in the same medium but containing 0.3 mg/ml of G418. In order to investigate the effect of androgen deprivation, cells were cultured in a medium containing 10% charcoal-stripped FBS (CS-FBS) instead of regular 10% FBS.

In Vitro Growth Assay

LNCaP and IL-6 over-expressing cells (LN-S15 and LN-S17) (10^4/well) were plated in 12-well plates in RPMI 1640 containing 10% FBS. After 2 days in regular culture medium with 10% FBS, cells were switched into RPMI 1640 medium containing either 10% FBS or 10% CS-FBS (Hyclone, UT). Three days later, cells were trypsinized and counted with a Coulter counter. For the IL-6 antibody test, 20 μg of IL-6 antibody (Sigma Chemicals Co.) was added per ml of culture medium.

Apoptosis ELISA Test

The ELISA kit was obtained from Roche Molecular Biochemicals (Indianapolis, IN) and the protocol was followed according to the manufacturer's instructions. For the preparation of samples, LNCaP and IL-6 over-expressing cells (LN-S15 and LN-S17) (10^4/well) were plated in 12-well plates in RPMI 1640 containing 10% FBS. After 2 days in regular culture medium with 10% FBS, cells were switched into a medium containing either 10% FBS or 10% CS-FBS (Hyclone). Three days later, cell lysates were obtained and cell death was analyzed and the absorbance values were normalized by the cell number.

Fluorescent Microscopic Studies

Cells (5×10^3) were plated in microslides in normal medium for 2 days and switched into a medium containing CS-FBS after washing. Three days later, cells were fixed in 8% paraformaldehyde for 15 min, washed and stained with Hoechst 33258 solution (5 μg/ml in PBS, 5 min). Cells were then washed with PBS, mounted and examined under fluorescent microscope.

Transfection

LNCaP, LN-S15, and LN-S17 cells were transiently transfected with 2 μg of either a dominant negative Stat3 (Stat3F) or a constitutively activated Stat3 mutant (Stat3c) using SuperFect Transfection Reagent (QIAGEN, Inc., Valencia, CA). Briefly, cells (1×10^6) were plated into 6-well plates and allowed to adhere for 24 hr. Cells were transfected with either Stat3F or Stat3c in serum-free medium for 4 hr, incubated with complete medium for 24 hr, and then were switched into a medium containing CS-FBS. Three days later, the cell lysates were prepared and used for the quantitation of apoptosis by the ELISA kit, growth assay, Western blot analysis, and electromobility shift assay (EMSA). For controls, the same amount of empty vector was used for transfection.

EMSA

Whole cell extracts were prepared and EMSA was performed as described previously [27]. For determination of the Stat3 DNA binding activity, whole cell extracts (20 μg) were incubated in a final volume of 20 μl (10 mM HEPES, pH 7.9, 80 mM NaCl, 10% glycerol, 1 mM DTT, 1 mM EDTA, 100 μg/ml poly(dIdC) with radiolabled double stranded Stat3 consensus binding motif 5′-GATCCTTCTGGGAATTCCTAGATC (Santa Cruz Biotechnologies, CA). The protein-DNA complexes were resolved on a 4.5% non-denaturing polyacrylamide gel at room temperature, and the results were autoradiographed using Molecular Imager FX System (Bio Rad, Hercules, CA).

Western Blot

Whole cell extracts were obtained, as described previously [19], and resolved in 8–12% SDS–PAGE depending on the molecular weight of the protein to be detected. After blocking overnight 4°C in 5% milk in PBS-0.1% Tween 20, membranes were incubated overnight with antibodies of cleaved caspase-9 (Cell Signaling Technology, MA), cleaved poly (ADP-ribose) polymerase (PARP) (Cell Signaling Technology), Bcl-x_L (Santa Cruz Biotechnologies), or phosphorylated Bad (Cell Signaling Technology). Following secondary antibody incubation, immunoreactive proteins were visualized with an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Buckinghamshire, England).

Statistical Analysis

Values were expressed as the mean ± SE. Statistical analyses were performed by one-way ANOVA, followed by the Student–Newman–Keuls test for multiple comparisons, with a $P < 0.05$ significant.

RESULTS

Overexpression of IL-6 in LNCaP Cells Confers Resistance to Androgen Deprivation-Induced Apoptosis

Previous studies have demonstrated that the androgen sensitive LNCaP cells express the IL-6 receptor, but express no detectable IL-6 protein [12–15,28]. To test the effect of IL-6 on LNCaP cell function, we ectopically expressed IL-6 by introduction of a full-length IL-6 cDNA into IL-6-negative LNCaP cells as described previously [13]. Two stable IL-6 independent transfectants (LN-S15 and LN-S17) were isolated that express high levels of IL-6 (2,465 and 2,743 pg/ml/10^6 cells, respectively) with a vector-alone control (neo). We previously demonstrated that the growth of androgen sensitive LNCaP cells in culture was reduced by about 50% after 48 hr in androgen-deprived charcoal-stripped serum condition compared with that in the normal serum condition [19]. However, in the IL-6 overexpression cells, there was only a 5–10% decrease in growth under these androgen-deprived conditions compared with growth in normal serum, suggesting that overexpression of IL-6 can enhance the growth of LNCaP cells in the androgen deprived condition in vitro.

Androgen deprivation triggers apoptosis in both normal and malignant androgen-dependent prostate epithelial cells. LNCaP cells are androgen sensitive human prostate cancer cells. To test whether LNCaP cells undergo programmed cell death in androgen deprived condition in vitro, and whether IL-6 can prevents apoptosis, LNCaP cells, LNCaP-neo, and IL-6 overexpression cells (LN-S15 and LN-S17) were cultured in RPMI 1640 with 10% FBS and then switched to RPMI 1640 with 10% CS-FBS in which physiological levels of androgen were deprived. Death was assessed 72 hr later through different techniques. The characteristic morphology of apoptosis was determined by staining with Hoechst 33258. Figure 1 shows apoptotic morphologies of the representative parental LNCaP and IL-6 overexpressing LN-S17 cells in androgen deprived conditions. A typical apoptotic morphology including chromatin condensation and nuclear fragmentation was clearly observed in the parental LNCaP and neo control cells cultured in the CS-FBS conditions, but not in IL-6-over-expressing cells (LN-S15 and LN-S17).

Apoptotic cell death was determined using the apoptosis specific ELISA assay to evaluate DNA fragmentation. As shown in Figure 2, parental LNCaP and neo control cells showed significant levels of apoptotic death in CS-FBS as compared to normal FBS ($P < 0.01$), whereas IL-6 over-expressing cells showed a significant lower level of apoptotic death as compared to parental LNCaP and neo control cells ($P < 0.01$).

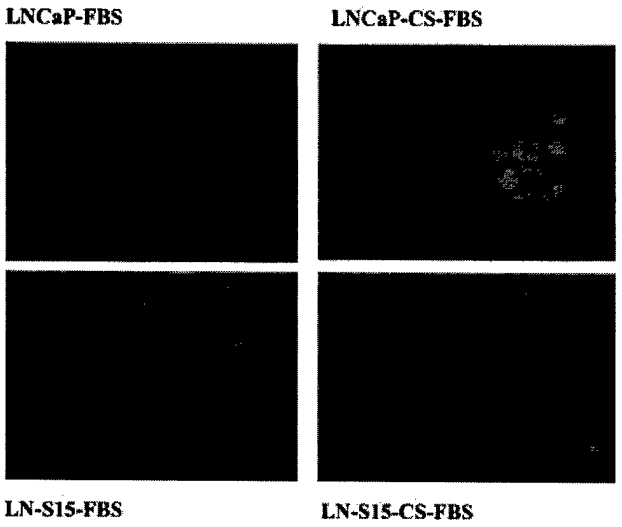

Fig. 1. Expression of interleukin-6 (IL-6) protects LNCaP cells against apoptosis induced by androgen deprivation. Parental LNCaP and IL-6 overexpressing LN-15 cells (LN-S15) were cultured in either normal FBS or androgen deprived CS-FBS conditions for 3 days. The cells were stained with Hoechst 33258 fluorescent dye and examined by fluorescent microscope. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Addition of IL-6 antibody to the cell culture media almost completely blocked the anti-apoptotic activity of IL-6 ($P < 0.01$), indicating that the anti-apoptotic effect was mediated specifically by IL-6.

Immunoblots using antibodies against several pro-apoptotic and anti-apoptotic proteins were performed to determine the effect of IL-6 on apoptosis. When apoptosis occurs, caspases are cleaved into active enzymes from inactive precursors and PARP is also cleaved. When parental LNCaP cells or neo control LNCaP cells were androgen deprived, elevated levels of the cleaved PARP and cleaved caspase-9 were detected (Fig. 3A). However, in IL-6 overexpressing LN-S15 and LN-S17 cells significantly lower levels of cleaved PARP and cleaved caspase-9 were observed as compared to the parental LNCaP cells or to the neo control cells when grown in androgen deficient

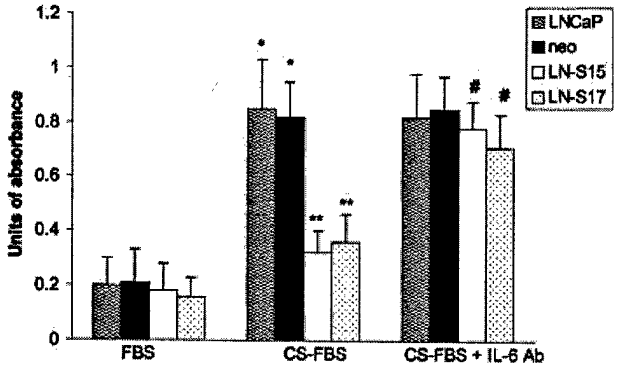

Fig. 2. Cell death analysis by a specific ELISA kit. Parental LNCaP, neo, and IL-6 overexpressing (LN-S15 and LN-S17) cells were cultured in either normal FBS, androgen deprived CS-FBS, or CS-FBS plus 20 μg/ml of IL-6 antibody conditions. Data are expressed as mean ± SE of four independent experiments. *, $P < 0.01$ compared with neo control cells cultured in normal FBS conditions; **, $P < 0.01$ compared with neo controls in CS-FBS conditions; #, $P < 0.01$ compared with LN-S15 and LN-S17 in the CS-FBS condition in the absence of the IL-6 antibody.

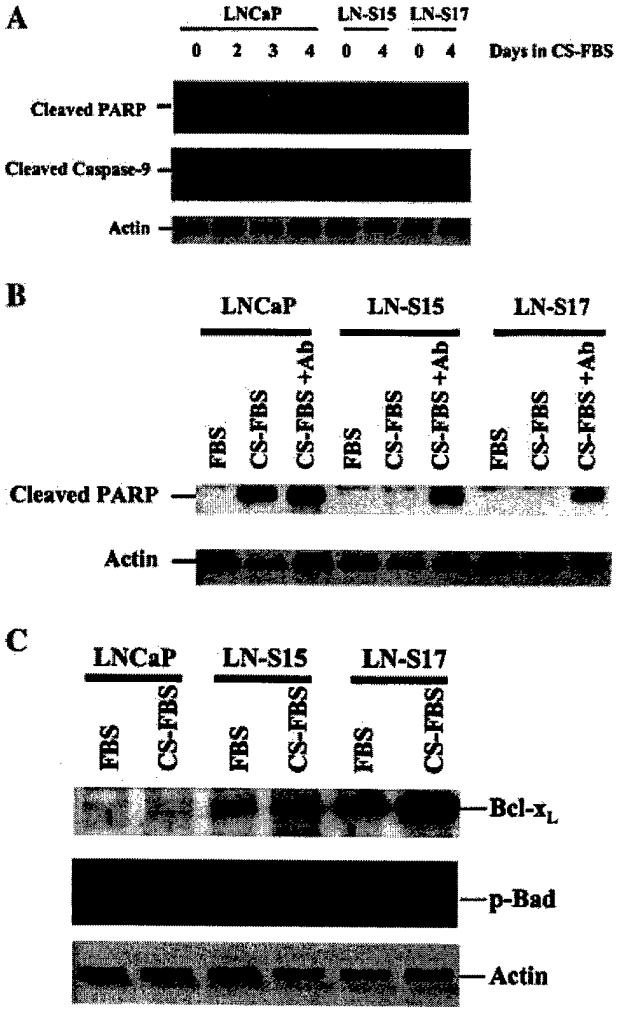

Fig. 3. Effect of IL-6 on the expression of pro-apoptotic and anti-apoptotic proteins in LNCaP cells in androgen deprivation conditions. Immunoblots were prepared from 40 μg of whole cell lysate from parental LNCaP, neo, and IL-6 overexpressing LNCaP clone LNSI5 and LN-SI7 cells cultured either in normal FBS or androgen deprived charcoal-stripped FBS (CS-FBS) conditions for 3 days as indicated. **A**: Immunoblots were analyzed with cleaved poly (ADP-ribose) polymerase (PARP) and cleaved caspase-9 antibodies. **B**: Twenty microgram per milliliter of IL-6 antibody were added to the androgen deprived charcoal stripped culture medium (CS-FBS-Ab), and whole cell lysate were immunoblotted with cleaved PARP antibody. **C**: Immunoblots were analyzed with Bcl-x_L and phospho-Bad antibodies.

conditions (Fig. 3A). Addition of IL-6 antibody to the IL-6 overexpressing LN-S15 and LN-S17 cell culture media significantly elevated the levels of cleaved PARP expression (Fig. 3B), further suggesting a role for IL-6 specifically. Concomitantly, the increase in anti-apoptotic protein was observed in LN-S15 and LN-S17 cells. When cells were grown in androgen-deficient media, the expression of Bcl-x_L and phosphorylated Bad proteins was significantly increased in the IL-6 overexpressing LN-S15 and LN-S17 cells compared to parental LNCaP cells (Fig. 3C). Collectively, these data demonstrate that LNCaP cells undergo apoptotic cell death upon androgen withdrawal, and that IL-6 protects these cells from androgen deprivation induced apoptosis.

Stat3 Activation Mediates Anti-Apoptotic Activity of IL-6

We have previously demonstrated that IL-6 significantly activates Stat3 in LNCaP cells both with androgen (normal FBS) and without androgen (CS-FBS) [19]. To elucidate the mechanism underlying this anti-apoptotic effect of IL-6, we investigated whether activation of Stat3 by IL-6 is responsible for this effect. We transfected a dominant-negative Stat3 mutant, Stat3F, into the LNCaP parental, neo, and IL-6 overexpressing cells. The dominant-negative Stat3 construct carries a phenylalanine substitution of the tyrosine residue at 705 that causes a reduction of the tyrosine phosphorylation of wild type Stat3 and inhibits the action of endogenous Stat3 [27,29,30]. As seen in Figure 4A, when Stat3F was transfected, the anti-apoptotic activity of IL-6 was abolished in IL-6 overexpressing LN-S15 and LN-S17 cells. When these cells were put into media without androgen, the survival rates were greater than 85%. In cells transfected with Stat3F, survival rates were less than 50%, a level observed in the parental LNCaP and neo control cells grown in the absence of androgen. The effect of transfecting expression of Stat3F on apoptosis was also examined by ELISA. As shown in Figure 4B, transient transfection of Stat3F into IL-6 overexpressing LN-S15 and LN-S17 cells resulted in a significant increase in apoptotic death ($P < 0.01$). Figure 4C demonstrated that the levels of Stat3 activity in these IL-6 overexpressing LN-S15 and LN-S17 clones were significantly decreased after the transient transfection of Stat3F. These studies confirm that blocking Stat3 activation reverses the anti-apoptotic activity of IL-6.

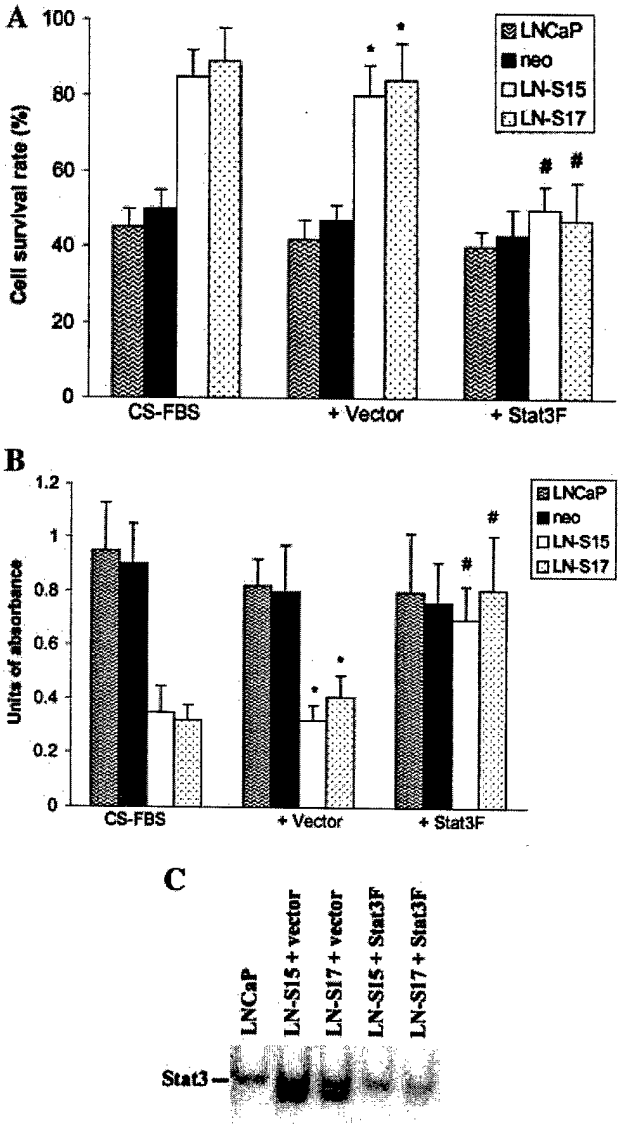

Fig. 4. Stat3F inhibits anti-apoptotic activity of IL-6. **A**: Stat3F inhibits IL-6 induced LNCaP cell survival in androgen deprived media. Parental LNCaP, neo, IL-6 overexpressing LN-S15 and LN-S17 cells were cultured in RPMI 1640 supplemented with 10% CS-FBS. The cells were then transiently transfected with equal amounts (2 μg) of either vector controls or Stat3F. The cell numbers were counted after 3 days in CS-FBS condition and cell survival values were expressed as % relative to the complete FBS. **B**: Stat3F blocks anti-apoptotic activity of IL-6 in androgen deprived conditions. Parental LNCaP, neo, IL-6 overexpressing LN-S15 and LN-S17 cells were cultured in RPMI 1640 supplemented with 10% CS-FBS. The cells were then transiently transfected with equal amounts (2 μg) of either vector controls or Stat3F. Quantitation of apoptosis by a special ELISA kit in these cells after cultured in androgen deprived CS-FBS for 3 days. Data are expressed as mean ± SE of four independent experiments. *, $P < 0.05$ compared with neo control in CS-FBS conditions; #, $P < 0.05$ compared with LN-S15 and LN-S17 transfected with vector control in the CS-FBS condition. **C**: Effect of Stat3F on Stat3 activity. IL-6 overexpressing LN-S15 and LN-S17 cells were transfected with either vector alone or Stat3F. After transfection, the cells were continuously cultured in androgen deprived condition for 3 days and the cell extracts were prepared. The Stat3 activity was analyzed using electromobility shift assay (EMSA) as described in "Materials and Methods."

Stat3 Activity Antagonizes Androgen-Deprivation Induced Death in LNCaP Cells

The anti-apoptotic activity of IL-6 from androgen deprivation appears to be mediated by Stat3 activation in LNCaP cells. We further investigated whether increased Stat3 activity might be sufficient to protect cells from androgen deprivation-induced apoptosis. To demonstrate a direct effect of enhanced Stat3 activity on LNCaP cell apoptosis induced by androgen deprivation, LNCaP cells were transfected with a constitutively activated Stat3 mutant, Stat3c, and vector control, respectively. Stat3c is a constitutively activated Stat3 (a mutant produced by substitution of the cysteine residues within the COOH-terminal loop of the SH2 domain of Stat3) that induces cellular transformation and tumor formation in nude mice [31]. Previous studies have demonstrated that ectopic expression of Stat3c into LNCaP cells enhanced Stat3 activation and promoted cell androgen independent growth [32]. After transfection with Stat3c, the cells were deprived of androgen for 72 hr, starting 12 hr after transfection, and the cell lysis was collected. Cell death was determined by the ELISA assay. As shown in Figure 5, overexpression of constitutively activated Stat3 in LNCaP cells resulted in significantly less cell death as compared to the vector control cells ($P < 0.01$) without androgen, and addition of the dominant-negative Stat3 (Stat3F) restored the level of cell death to that of the vector control, suggesting that the constitutive activation of Stat3 is sufficient to protect LNCaP undergoing apoptosis induced by androgen deprivation.

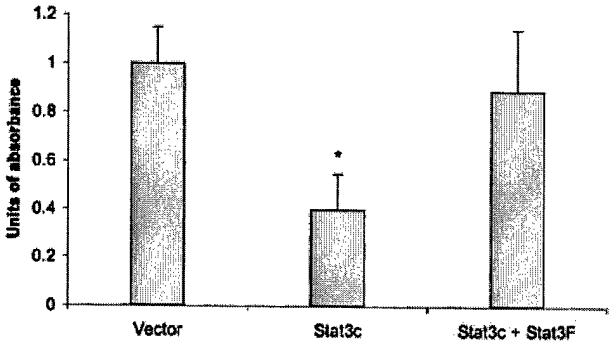

Fig. 5. Constitutively activated Stat3 protects LNCaP cells apoptosis induced by androgen deprivation. LNCaP cells were cultured in RPMI 1640 supplemented with 10% CS-FBS. The cells were then transiently transfected with equal amounts (2 μg) of either vector controls or constitutively activated Stat3c or Stat3c plus Stat3F. Quantitation of apoptosis by a special ELISA kit was performed after cultured in androgen deprived CS-FBS for another 3 days. Data are expressed as mean ± SE of four independent experiments. *, $P < 0.05$ compared with vector control in CS-FBS conditions.

DISCUSSION

In this study, we have investigated the molecular mechanisms by which IL-6 promotes androgen independent progression of prostate cancer cells. We demonstrated that IL-6 protects androgen sensitive LNCaP cells from androgen deprivation induced apoptosis through activation of Stat3 signaling pathway.

We previously demonstrated that overexpression of IL-6 in androgen sensitive LNCaP cells enhances androgen independent growth in vitro and in vivo [19]. To understand the mechanism of IL-6 induced androgen independent growth of LNCaP cells, we analyzed the effect of IL-6 on apoptosis induced by androgen deprivation. Several lines of evidence from this study demonstrate that overexpression of IL-6 can protect androgen deprivation-induced apoptosis in LNCaP cells. First, overexpression of IL-6 rescued LNCaP cells from cell death induced by androgen withdrawal. Second, LNCaP cells underwent apoptosis 72 hr androgen was removed, an outcome is largely absent in clones overexpressing IL-6. Third, IL-6 overexpressing cells resulted in a significant decrease in the expression of pro-apoptotic proteins such as cleaved PARP and cleaved caspase-9, and an increase in the expression of the anti-apoptotic proteins Bcl-x_L and phosphorylated Bad compared to the parental LNCaP cells. Forth, addition of IL-6 antibody completely abolished the anti-apoptotic activity of IL-6, suggesting that anti-apoptosis is specifically mediated by IL-6.

IL-6 has been implicated in the modulation of growth and differentiation in many malignant tumors and is associated with poor prognosis in several solid and hematopoietic neoplasms such as renal cell carcinoma, ovarian cancer, lymphoma, and melanoma [33]. The role of IL-6 in prostate cancer development and progression has been a subject of intensive investigation. The expression of IL-6 and its receptor has been consistently demonstrated not only in human prostate cancer cell lines but more importantly in human prostate carcinoma and benign prostate hyperplasia obtained directly from patients [8–10]. Clinically, the levels of IL-6 in the sera are significantly elevated in the patients with hormone refractory and metastatic prostate cancer compared to hormone sensitive prostate cancer [4,11]. Multiple studies have demonstrated that IL-6 is elevated in the sera of patients with metastatic prostate cancer and that the levels of IL-6 correlate with tumor burden as well as serum PSA or clinically evident metastases [4,11]. Collectively, these clinical data suggest that elevated IL-6 levels are associated with prostate cancer progression to an androgen-independent phenotype.

In addition to the clinical data on the role of IL-6 in androgen independent prostate cancer, experimental

studies demonstrate that IL-6 plays a critical role in prostate cancer cell growth and differentiation. IL-6 can function as a paracrine growth factor for the human LNCaP androgen-sensitive prostate cancer cells and an autocrine growth factor for the human DU145 and PC-3 androgen-insensitive prostate cancer cells [12]. IL-6 can activate AR-mediated gene expression by activation of the AR through a Stat3 pathway in LNCaP cells [13,16,17]. Our previous studies demonstrated that overexpression of IL-6 increases PSA mRNA expression and enhances AR activation in LNCaP cells [19]. In addition, overexpression of IL-6 promotes androgen independent growth of androgen sensitive LNCaP cells in vitro and in vivo [19]. These studies demonstrate that IL-6 activates AR signaling in a ligand-independent manner and induces a synergistic AR response with very low concentrations of androgen.

Accumulating evidence has demonstrated that abnormal AR signaling has contributed to prostate cancer androgen independent growth. Several reports suggested that the AR can be activated by growth factors and cytokines to display enhanced activity in the presence of androgen or to function even in the absence of androgen [34–37]. Recently, results from a number of groups demonstrated that IL-6 activates AR-mediated gene expression by activation of the AR through a Stat3 pathway in LNCaP cells [13,14,16,17]. Induction of neuroendocrine differentiation has also been suggested in the androgen independent prostate cancer cells [38,39]. IL-6 can induce LNCaP cell neuroendocrine differentiation mediated by the activation of Stat3 and MAPK signalings [40–42]. Androgen withdrawal triggers apoptosis in both normal and malignant androgen-dependent prostate epithelial cells. However, androgen-refractory prostate cancer cells do not undergo apoptosis [1]. Thus alteration of apoptotic signaling pathways should be critical for the survival of androgen-refractory prostate cancer cells. In this study, we demonstrate that IL-6 protects LNCaP cells from undergoing apoptotic death induced by androgen deprivation.

The effect of IL-6 on cell proliferation, differentiation, and survival is mediated by differential activation of several major signaling pathways including JAK-STAT, MAPK, and PI3K-Akt. The question arises as to what is the mechanism of anti-apoptosis induced by IL-6 in LNCaP cells. Apoptosis can be induced in response to various cytotoxic stimuli including cytokines and growth factors. These stimuli activate a series of tightly controlled intracellular signals including Stat3. The role of Stat3 in the protection of apoptosis has been already suggested in many cancer cells including prostate [43]. In U266 myeloma cells, constitutive activation of Stat3 signaling confers resistance to apoptosis [44]. In addition, Stat5 and Stat3 have also been demonstrated to activate the Bcl-x_L expression directly [44,45]. In this study, we have clearly shown that the anti-apoptotic activity of IL-6 is mediated mainly by Stat3 signaling. We demonstrated that IL-6 overexpressing cell clones are protected from androgen-deprivation induced apoptosis through activation of Stat3 signaling. This protective effect of IL-6 was reversed by the expression of a dominant-negative Stat3 mutant, Stat3F. Furthermore, ectopic expression of a constitutively active Stat3 antagonized androgen deprivation-induced cell death of LNCaP cells.

In conclusion, we demonstrate that overexpression of IL-6 protects androgen sensitive LNCaP prostate cancer cells from apoptotic death induced by androgen deprivation through activation of Stat3 signaling pathway. Since both IL-6 levels are significantly elevated in hormone refractory prostate cancer and Stat3 activity is elevated in prostate cancer [46], targeting IL-6/Stat3 signaling may be of therapeutic value in the treatment of androgen independent prostate cancer.

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[CANCER RESEARCH 64, 19–22, January 1, 2004]

Appendix 3

Advances in Brief

Prostate Specific Antigen Expression Is Down-Regulated by Selenium through Disruption of Androgen Receptor Signaling

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Abstract

A previous controlled intervention trial showed that selenium supplementation was effective in reducing the incidence of prostate cancer. Physiological concentrations of selenium have also been reported to inhibit the growth of human prostate cancer cells *in vitro*. The present study describes the observation that selenium was able to significantly down-regulate the expression of prostate-specific antigen (PSA) transcript and protein within hours in the androgen-responsive LNCaP cells. Decreases in androgen receptor (AR) transcript and protein followed a similar dose and time response pattern upon exposure to selenium. The reduction of AR and PSA expression by selenium occurred well before any significant change in cell number. With the use of a luciferase reporter construct linked to either the PSA promoter or the androgen responsive element, it was found that selenium inhibited the *trans*-activating activity of AR in cells transfected with the wild-type AR expression vector. Selenium also suppressed the binding of AR to the androgen responsive element site, as evidenced by electrophoretic mobility shift assay of the AR-androgen responsive element complex. In view of the fact that PSA is a well-accepted prognostic indicator of prostate cancer, an important implication of this study is that a selenium intervention strategy aimed at toning down the amplitude of androgen signaling could be helpful in controlling morbidity of this disease.

Introduction

Prostate cancer is the second most common cancer as well as the second most common cause of cancer death in men in the United States. Every year, there are ~190,000 new cases and 30,000 deaths from prostate cancer (1). Age is a major risk factor; the incidence is 1 in 53 for men in their 50s but 1 in 7 for men from 60 to 80 years of age. A chemopreventive modality that can suppress or delay the clinical symptoms of prostate cancer would be well suited for preserving the quality of life in high-risk elderly men. In a previous randomized, placebo-controlled cancer prevention trial in which prostate cancer was evaluated as a secondary end point (974 of the 1312 subjects in the cohort were men), supplementation with a nutritional dose of selenium was found to reduce prostate cancer incidence by 50% (2, 3). Recent studies by Dong *et al.* (4) showed that selenium inhibited human prostate cancer cell growth, blocked cell cycle progression at multiple transition points, and induced programmed cell death. Prostate specific antigen (PSA) is a gene known to be under the control of the androgen receptor (AR) and is a well-accepted marker for the diagnosis and prognosis of prostate cancer. In view of the clinical observation of the effectiveness of selenium in prostate cancer prevention, it is reasonable to believe that selenium might be able to reduce the expression of PSA. If confirmed, this attribute obviously has the advantage of forecasting the responsiveness to selenium intervention. In this report, we describe a series of experiments that were designed to test the hypothesis that selenium is capable of down-regulating PSA through a mechanism of attenuating the functional intensity of the AR signal transduction pathway.

As discussed previously (4), cultured prostate cells respond poorly to selenomethionine (a commonly used selenium reagent) and only when it is present at supraphysiological levels in the medium. A plausible explanation is that prostate cells have a low capacity in metabolizing selenomethionine to methylselenol (CH_3SeH), which is believed to be the active species for selenium chemoprevention (5). This process normally takes place in the liver and kidney. For this reason, methylseleninic acid (CH_3SeO_2H, abbreviated to MSA) was developed by Ip *et al.* (6) specifically for *in vitro* experiments. Once taken up by cells, MSA is readily reduced by glutathione and NADPH to methylselenol (which is rather unstable in itself) via a non-enzymatic reaction. The cellular and molecular responses of prostate cells to physiological concentrations of MSA have been documented in a number of publications (4, 7–10). Thus, we believe we have the right tool to study the effect of selenium on AR signaling.

Materials and Methods

Selenium Reagent, Prostate Cell Culture, and Cell Growth Analysis. MSA was synthesized as described previously (6). The LNCaP human prostate cancer cells were obtained from American Type Culture Collection (Manassas, VA). The cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 units/ml of penicillin, and 100 μg/ml of streptomycin (11). In some experiments, cells were cultured in an androgen-defined condition by using charcoal-stripped FBS in the presence of 10 nM R1881 (a potent synthetic androgen). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was performed 24, 48, or 72 h after MSA treatment as described previously (11). For the quantitative determination of AR and PSA transcripts and proteins, cells were exposed to MSA for much shorter periods of time, usually 24 h or less. Total RNA and protein were isolated using the TRIzol reagent (Invitrogen, Carlsbad, CA).

Real-Time Reverse Transcription-PCR. First-strand cDNA was synthesized from 100 ng of total RNA by SuperScript II reverse transcriptase (Invitrogen) following the manufacturer's protocol. The PCR primers and TaqMan probes for β-actin, AR, and PSA were Assays-on-Demand products from Applied Biosystems. Two μl of first-strand cDNA were mixed with 25 μl of 2× Taqman Universal PCR Master Mix (Applied Biosystems) and 2.5 μl of 20× primers/probe mixture in a 50-μl final volume. Temperature cycling and real-time fluorescence measurement were performed using an ABI prism 7700 Sequence Detection System (Applied Biosystems). The PCR conditions were as follows: an initial incubation at 50°C for 2 min, then a denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min.

The relative quantitation of gene expression was performed using the comparative C_T ($\Delta\Delta C_T$) method (12). Briefly, the threshold cycle number (C_T) was obtained as the first cycle at which a statistically significant increase in fluorescence signal was detected. Data normalization was carried out by subtracting the C_T value of β-actin from that of the target gene. The $\Delta\Delta C_T$ was calculated as the difference of the normalized C_T values (ΔC_T) of the treatment

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Notes: Y. Dong and S. O. Lee contributed equally to this work.

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and control samples: $\Delta\Delta C_T = \Delta C_{T\ treatment} - \Delta C_{T\ control}$. Finally, $\Delta\Delta C_T$ was converted to fold of change by the following formula: Fold of change = $2^{-\Delta\Delta C_T}$.

Western Blot Analysis. Details of the procedure were described previously (4). Immunoreactive bands were quantitated by volume densitometry and normalized against either glyceraldehyde-3-phosphate dehydrogenase or α-actin. The following monoclonal antibodies were used (source): anti-glyceraldehyde-3-phosphate dehydrogenase (Chemicon, Temecula, CA), anti-α-actin (Sigma Chemical Co., St. Louis, MO), anti-AR (BD Transduction Laboratory, San Jose, CA), and anti-PSA (Santa Cruz Biotechnology, Santa Cruz, CA).

Transfection and Luciferase Assay. An aliquot of 3×10^5 cells was placed in a 6-well plate and then transfected with a total amount of 5 μg of DNA using Superfect (Qiagen, Valencia, CA) according to the manufacturer's instructions. Two different constructs were evaluated: the PSA promoter-luciferase reporter plasmid (13) and the androgen responsive element (ARE)-luciferase reporter plasmid (14). The total amount of plasmid DNA was normalized to 5 μg/well by the addition of empty plasmid. The DNA/liposome mixture was removed 3 h later, and cells were treated with different concentrations of MSA in the presence of 10 nM R1881. Cell extracts were obtained after 24 h, and luciferase activity was assayed using the Luciferase Assay System (Promega, Madison, WI). Protein concentration in cell extracts was determined by the Coomassie Plus protein assay kit (Pierce, Rockford, IL). Luciferase activities were normalized by the protein concentration of the sample. All transfection experiments were performed in triplicate wells and repeated at least four times.

Nuclear Lysate Preparation. Nuclear protein extract was prepared as described previously (15). Cells were harvested, washed with PBS twice, and resuspended in a hypotonic buffer [10 mM HEPES-KOH (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, and 0.1% NP40] and incubated on ice for 10 min. Nuclei were precipitated with 3000 × *g* centrifugation at 4°C for 10 min. After washing once with the hypotonic buffer, the nuclei were lysed in a lysis buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 1% Triton X-100] and incubated on ice for 30 min. The nuclear lysate was precleared by 20,000 × *g* centrifugation at 4°C for 15 min. Protein concentration was determined by the Coomassie Plus protein assay kit.

Electrophoretic Mobility Shift Assay (EMSA). A quantity of 20 μg of nuclear protein extract was incubated in a 20-μl solution containing 10 mM HEPES (pH 7.9), 80 mM NaCl, 10% glycerol, 1 mM DTT, 1 mM EDTA, 100 μg/ml poly(deoxyinosinic-deoxycytidylic acid), and the radiolabeled double-stranded AR consensus binding motif 5′-CTAGAAGTCTGGTACAGGGT-GTTCTTTTTGCA-3′ (Santa Cruz Biotechnology). The protein-DNA complexes were resolved on a 4.5% nondenaturing polyacrylamide gel containing 2.5% glycerol in 0.25× Tris-borate EDTA at room temperature, and the results were autoradiographed. Quantitation of AR DNA-binding activity in the "protein-DNA" bandshift was measured using the Molecular Imager FX System (Bio-Rad, Hercules, CA). For the supershift experiment, 20 μg of cell extract protein were incubated with the monoclonal AR antibody (Santa Cruz Biotechnology) for 1 h at 4°C before incubation with the radiolabeled probe.

Results

MSA Inhibits LNCaP Cell Growth in a Dose- and Time-dependent Manner. Table 1 shows the results of the effect of MSA treatment on cell growth. The data are expressed as percentages of the untreated control. A concentration of 2.5 μM MSA produced essentially no change, even after 3 days of treatment. Increasing the concentration of MSA to 5 μM inhibited cell growth by about 25%, but the effect was not observed until the 72-h time point. The same magnitude of growth inhibition was observed at 24 h with 10 μM MSA, and by 72 h, there were 50% fewer cells compared with the untreated culture. The experiment therefore established the dose and time response to MSA with respect to growth inhibition. This information is important because the down-regulation of PSA and AR by selenium occurs well before the onset of growth inhibition (see below).

Table 1 *Effect of MSA on the accumulation of LNCaP cells*[a]

Treatment	Treatment duration (h)[b]		
	24	48	72
MSA (μM)			
2.5	102.5 ± 4.0	106.6 ± 6.2	102.5 ± 1.9
5	93.7 ± 3.1	96.4 ± 2.9	72.6 ± 1.9[c]
10	77.1 ± 8.4[c]	61.1 ± 1.7[c]	55.4 ± 3.7[c]

[a] As a percentage of untreated control.
[b] Results are expressed as mean ± SE (n = 4 independent experiments).
[c] Significantly different compared with the corresponding control value ($P < 0.05$).

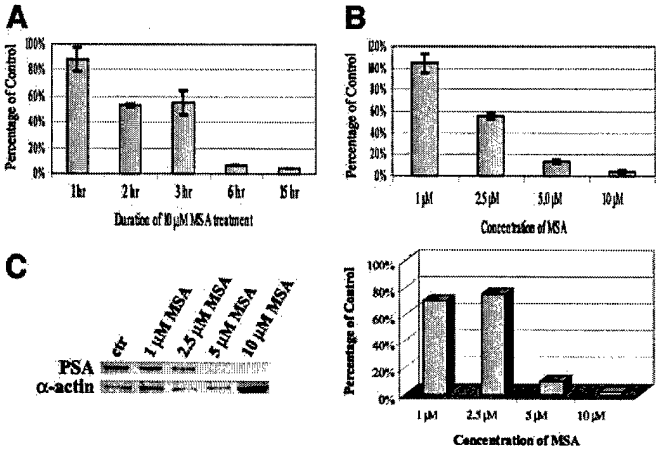

Fig. 1. Effect of MSA on PSA expression. *A* and *B*, changes in PSA mRNA, as determined by quantitative RT-PCR, as a function of time of treatment with MSA or as a function of MSA concentration. With the exception of the 1-h and the 1 μM MSA data points, the remaining data points are significantly different ($P < 0.01$) from the control, which is set as 100%. *Bars*, SE. *C*, Western blot data of changes in PSA protein level as a function of different concentrations of MSA (*left side*) and normalized quantitative changes compared with the control value of 100% (*right side*).

MSA Suppresses PSA mRNA and Protein Expression in LNCaP Cells. The modulation of PSA mRNA by MSA was assessed quantitatively by real-time RT-PCR. Cells were treated with 10 μM MSA for various lengths of time; the PSA results are shown in Fig. 1*A*. A marked decrease in PSA mRNA was detected as early as 2 h after exposure to MSA; the mRNA level dropped to <10% of the control value by 6 and 15 h. As shown in Fig. 1*B*, the depression of PSA mRNA was dependent on the concentration of MSA in the range between 2.5 and 10 μM; the assay was performed after exposure to MSA for 15 h. As little as 2.5 μM MSA reduced PSA mRNA level by 40%. This level of MSA had no effect on cell growth. Even with 10 μM MSA, the near complete elimination of PSA mRNA expression occurred before there was any detectable change in growth. The decrease in PSA protein level by MSA at 15 h, as determined by Western blot analysis, is shown in Fig. 1*C*, *left panel*. The right panel shows the normalized quantitative changes compared with the control value of 100%. Small decreases in PSA protein were evident with 1 or 2.5 μM MSA. At 5 or 10 μM MSA, the level of PSA protein became very low or hardly detectable. The experiments described in Fig. 1 were done with cells cultured in 10% FBS. In addition, we carried out another set of experiments with cells cultured in charcoal-stripped FBS containing 10 nM R1881 (a potent synthetic androgen). The down-regulation of PSA mRNA by MSA, as a function of dose and time, was qualitatively similar to that observed with the FBS culture (data not shown).

MSA Suppresses AR mRNA and Protein Expression in LNCaP Cells. The expression of PSA is known to be regulated by AR, which is a ligand-activated transcription factor. Our next step was to investigate the expression of AR mRNA in response to MSA by real-time RT-PCR. Fig. 2*A* shows the time course of response to 10 μM MSA. Within the first hour, there was a 50% decrease in AR mRNA. The transcript level continued to drop down to 20% or below with longer

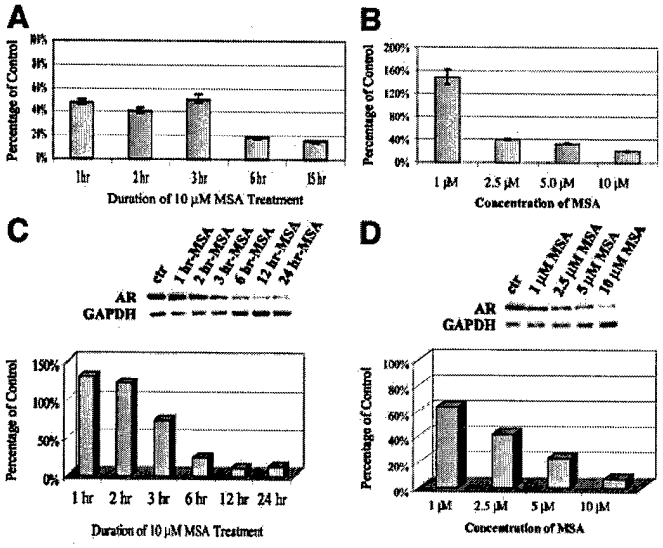

Fig. 2. Effect of MSA on AR expression. *A* and *B*, change in AR mRNA, as determined by quantitative RT-PCR, as a function of time of treatment with MSA or as a function of MSA concentration. All of the data are significantly different ($P < 0.01$) from the control, which is set as 100%. *Bars*, SE. *C*, Western blot data of changes in AR protein level as a function of time of treatment with 10 μM MSA. *D*, Western blot data of changes in AR protein level as a function of different concentrations of MSA. *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

treatment with MSA. The dose response to MSA is shown in Fig. 2*B*; these assays were done at the 15-h time point. Interestingly, 1 μM MSA actually increased slightly the level of AR mRNA. However, raising the concentration of MSA to 2.5 μM or above caused a very significant depression of the AR transcript to 40% or less of the control value. We next examined AR protein expression in response to 10 μM MSA. As shown in Fig. 2*C*, MSA down-regulated AR protein level progressively as a function of time over a period of 24 h. Initially, the reduction in protein appeared to lag behind the reduction in transcript by at least 2–3 h. The delay in response might be reflective of the time needed for protein turnover. Fig. 2*D* shows the effect of different concentrations of MSA on expression of the AR protein. MSA produced a graded suppression of the AR protein in a dose-dependent manner. In general, the changes in protein level were consistent with the real-time RT-PCR results with the exception of the 1 μM MSA data point.

MSA Inhibits AR *trans*-Activating Activity. LNCaP cells have a mutant but functional AR. In an attempt to determine the ability of MSA to interfere with AR *trans*-activating activity, we transiently transfected LNCaP cells with an expression vector for the wild-type AR and the PSA promoter-luciferase reporter plasmid. This region of the PSA regulatory element contains the promoter and enhancer and has been demonstrated to be responsive to androgen stimulation (13). As shown in Fig. 3*A*, MSA inhibited the luciferase reporter in a dose-dependent manner. Thus, the PSA promoter activity was decreased by 50, 67, 93, or 96% in the presence of 1, 2, 5, or 10 μM MSA, respectively.

Activated AR exerts its function by binding to the ARE site. Because the PSA promoter contains many regulatory elements in addition to the ARE, one could argue that the decrease in PSA promoter activity might not necessarily be attributable to a change in AR *trans*-activating activity. To address this issue, we transiently transfected LNCaP cells with an expression vector for the wild-type AR and the ARE-luciferase reporter plasmid. This construct contains three repeats of the ARE region ligated in tandem to the luciferase reporter (14). As shown in Fig. 3*B*, the ARE-luciferase activity was inhibited by 50, 60, or 75% in the presence of 1, 2, or 5 μM MSA, respectively. The result obtained with 10 μM MSA was similar to that with 5 μM MSA.

MSA Decreases Binding of AR to ARE. To determine whether MSA might reduce the DNA binding activity of the AR protein to the ARE, we performed EMSA using radiolabeled oligonucleotides of the ARE with nuclear extract from LNCaP cells treated for 30 min with various concentrations of MSA. As shown in Fig. 4, *A* and *B*, a decrease in AR-ARE complex formation was evident with MSA treatment compared with the untreated control. We can rule out the reduced availability of the AR protein as a contributing factor, because there was no change in AR protein after only 30 min of

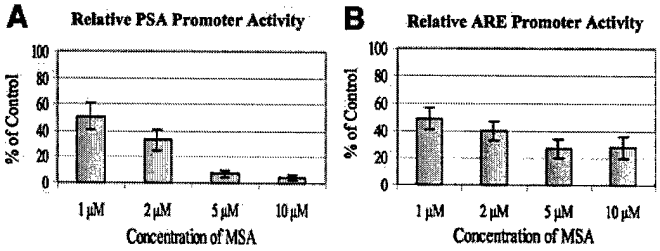

Fig. 3. Effect of MSA on PSA promoter activity (*A*) and ARE promoter activity (*B*). The cells were cultured in charcoal-stripped FBS containing 10 nM R1881. The results are expressed as percentages of untreated control. All of the data points are significantly different ($P < 0.01$) from the control value. *Bars*, SE.

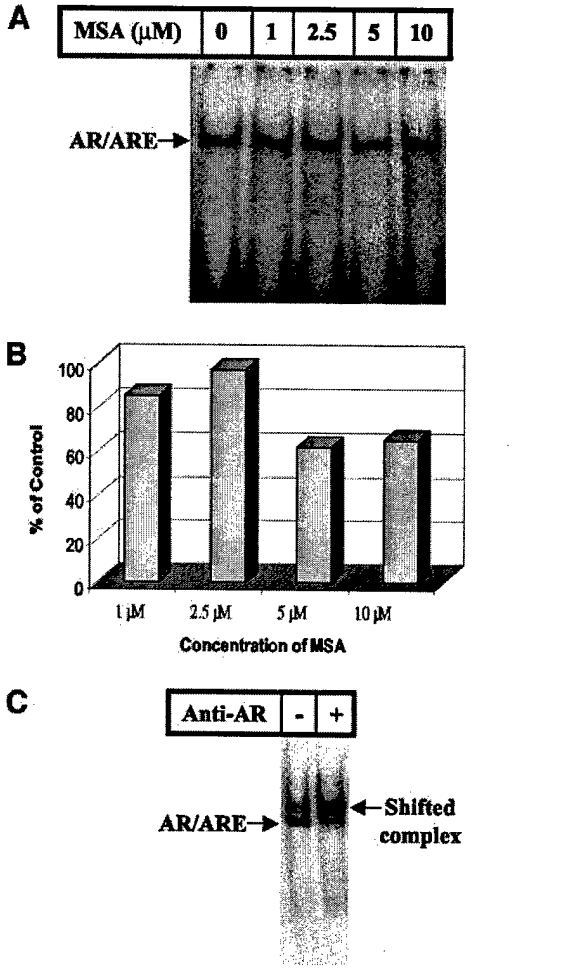

Fig. 4. *A*, EMSA results of AR binding to ARE as a function of different concentrations of MSA. *B*, quantitative determination of the EMSA results. *C*, supershift of the AR/ARE complex with antibody against AR.

treatment with MSA (see Fig. 2*C*). The specificity of the AR-ARE complex was demonstrated by the supershift assay using an antibody against AR (Fig. 4*C*).

Discussion

This report is the first to show that a selenium metabolite is able to down-regulate the expression of PSA in human prostate cancer cells via a mechanism involving disruption of the androgen signal transduction pathway. On the basis of the information from this study, selenium decreases AR transcript and protein and inhibits AR *trans*-activating activity. Selenium can also diminish the binding of AR to the ARE site. However, we cannot at this time distinguish whether this is attributable to a block in nuclear translocation of the activated AR or a physical interference of AR association with the ARE through modulation of other co-regulators. These various possibilities will be investigated systematically in the future. It is noteworthy that the reduction in AR and PSA expression occurs at least 20 h before any significant decrease in cell number. This kind of bellwether change at the molecular level might be one of the causes underlying the sensitivity of prostate cells to selenium treatment.

In a recent paper, Bhamre *et al.* (16) reported that although supraphysiological levels of selenomethionine inhibited LNCaP cell growth, selenomethionine did not specifically affect the production of PSA when the results were normalized to the decreased number of viable cells. As explained in the "Introduction," selenomethionine is not a suitable selenium reagent for cell biology studies *in vitro*, because it is poorly metabolized by cultured epithelial cells to the active monomethylated intermediate. Not surprisingly, many cellular and molecular events that are normally sensitive to modulation by physiological levels of MSA (4, 7–11) respond very sluggishly to selenomethionine, and only when it is present at excessively high levels in the medium. Thus, the discrepancy between our study and that of Bhamre *et al.* (16) can be reconciled by the differences in biochemical reactivity between MSA and selenomethionine.

The clonal expansion of prostate cancer at the early stage is mostly dependent on androgen stimulation. A selenium intervention strategy aimed at dampening the amplitude of androgen signaling could be helpful for controlling prostate cancer in high-risk men. PSA is a well-accepted diagnostic and prognostic biomarker of prostate cancer progression. The down-regulation of PSA by selenium therefore has significant clinical implication. In patients treated with selenium, the monitoring of PSA in the circulation could potentially be evaluated as a barometer to gauge the efficacy of intervention. The benefit might also be extended to the prevention of relapses after endocrine therapy. Recurrent prostate cancer is generally hormone refractory, although the expression of AR is maintained regardless of the clinical stage of the disease (17, 18). The fact that PSA continues to be produced by the pathologically advanced cancer suggests that the AR signal transduction pathway is still intact. Several hypotheses have been proposed to explain this phenomenon. Mutations of the AR may enable cells to be sensitized by very low levels of androgens, or perhaps even by non-androgen steroids (19). Alternatively, the receptor may become promiscuous and can be activated by non-steroidal growth factors and cytokines (20). Prostate cancer may also adapt to androgen deprivation by increasing the expression of AR through gene amplification (21, 22). We have developed a LNCaP subline that is not responsive to androgen but is capable of producing a copious amount of PSA. We are planning to use this cell model to further investigate the role of selenium in AR function when the presence of androgen is no longer required.

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